THE

BIOCHEMICAL JOURNAL

CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, MANAGER
LONDON: FETTER LANE, E.C. 4

H. K. LEWIS & CO., LTD., 136, GOWER STREET, LONDON, W.C. 1
WHELDON & WESLEY, LTD., 28, ESSEX STREET, LONDON, W.C. 2
CHICAGO: THE UNIVERSITY OF CHICAGO PRESS
(AGENT FOR THE UNITED STATES AND CANADA)
BOMBAY, CALCUTTA, MADRAS: MACMILLAN & CO., LTD.
TOKYO: THE MARUZEN-KABUSHIKI-KAISHA

All rights reserved

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

Dr G. BARGER	Prof. F. KEEBLE
Prof. V. H. BLACKMAN	Prof. B. MOORE
Mr J. A. GARDNER	Prof. W. RAMSDEN
Prof. F. G. HOPKINS	Dr E. J. RUSSELL

VOLUME XV 1921

CAMBRIDGE

AT THE UNIVERSITY PRESS

1921

Vol. XV, No. 1 1921

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

DR G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
PROF. F. G. HOPKINS
PROF. F. KEEBLE
PROF. B. MOORE
PROF. W. RAMSDEN
DR E. J. RUSSELL

CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C. 4

H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
WHELDON AND WESLEY, Ltd., 28, Essex Street, Strand, London, W.C. 2
CHICAGO: The University of Chicago Press
(Agent for the United States and Canada)
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1921

Price Twenty Shillings net

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

The annual subscription is 35*s*., which includes a copy of the Journal, and becomes due on January 1st. Further information may be obtained on application to the Hon. Sec., Dr J. C. Drummond, University College, London, W.C. 1, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. W. M. Bayliss, F.R.S., University College, W.C. 1, or to Prof. A. Harden, F.R.S., Lister Institute, Chelsea Gardens, S.W. 1. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3 net per volume (post free) payable in advance) should be addressed to Mr C. F. Clay, Cambridge University Press, Fetter Lane, London, E.C. 4. For prices of back numbers and volumes see p. 4 of this wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agents for the sale of the *Biochemical Journal* in the United States of America and has authorised them to fix the following subscription price. $12.00 net.

Notice to Contributors.

1. Contributors receive 50 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912, 1), *Zeitsch. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912, 1]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

Mad.
Faxon.

CONTENTS

No. 1

PAGE

OBITUARY NOTICE. Percival Spencer Umfreville Pickering . . . 1

I. The Action of Sodium Hydroxide upon Coagulation of Fibrinogen. By J. O. W. BARRATT. (With two figures) 4

II. The Production of Carbon Monoxide by the Action of Alkaline Hypohalogenites on Urea. By W. H. HURTLEY 11

III. The Effect on Kittens of a Diet deficient in Animal Fat. By H. M. M. MACKAY. (With four figures) 19

IV. The Effect of a Diet deficient in Animal Fat on the Bone Tissue (Rib Junctions) of Kittens. By F. M. TOZER 28

V. Comparison of the Growth-Promoting Properties for Guinea-Pigs of Certain Diets, consisting of Natural Foodstuffs. By E. M. HUME . . 30

VI. A Human Enterolith containing Choleic Acid. By H. S. RAPER 49

VII. The Nephelometric Estimation of Quinine in Blood. By H. W. ACTON and H. KING 53

VIII. Investigations on the Nitrogenous Metabolism of the Higher Plants. Part I. The Isolation of Proteins from Leaves. By A. C. CHIBNALL and S. B. SCHRYVER 60

IX. Studies on the Cycloclastic Power of Bacteria. Part II. A Quantitative Study of the Aerobic Decomposition of Tryptophan and Tyrosine by Bacteria. By H. RAISTRICK and A. B. CLARK 76

X. Determination of the Minimum Doses of some fresh Citrus Fruit Juices which will protect a Guinea-Pig from Scurvy, together with some Observations on the Preservation of such Juices. By A. J. DAVEY . . 83

XI. Glyoxalase in Avian Beriberi. By G. M. FINDLAY . . . 104

XII. Oxidising Enzymes. IV. The Distribution of Oxidising Enzymes among the Higher Plants. By M. W. ONSLOW 107

XIII. Oxidising Enzymes. V. Further Observations on the Oxidising Enzymes of Fruits. By M. W. ONSLOW 113

XIV. The Extraction of Melanin from Skin with Dilute Alkali. By W. J. YOUNG 118

XV. Constituents of the Bark of *Zanthoxylum macrophyllum*, Oliver. By J. A. GOODSON 123

XVI. The Antiscorbutic Value of Dried Milk. By H. JEPHCOTT and A. L. BACHARACH. (With six figures) 129

XVII. The Effect of Diet on Mammary Secretion. By G. A. HARTWELL. (With eight figures) 140

PAGE

XVIII. Investigation of the Anti-scorbutic Value of Full Cream Sweetened Condensed Milk by Experiments with Monkeys. By E. M. Hume 163

XIX. Note on the Permeability of the Red Corpuscles for Amino-Acids. By S. Kozawa and N. Miyamoto 167

XX. The Estimation of Blood Sugar. By E. Ponder and L. Howie 171

XXI. The Effect of Acetaldehyde and Methylene Blue on the Fermentation of Glucose and Fructose by Yeast-Juice and Zymin in presence of Phosphate and Arsenate. By A. Harden and F. R. Henley. (With five figures) 175

No. 2

XXII. A Comparative Investigation of the Corresponding Proteins of Cow and Ox Serum, Cow's Colostrum and Cow's Milk by the Method of Protein Racemisation. By H. E. Woodman. (With two diagrams) . . 187

XXIII. On the Theory of the Barcroft Differential Blood-gas Apparatus. By T. R. Parsons 202

XXIV. On the Physiology of the Silkworm. By A. P. Jameson and W. R. G. Atkins 209

XXV. The Influence of Reaction on Colour Changes in Tryptophan Solutions. By E. C. V. Mattick and R. S. Williams 213

XXVI. Methods of Measuring the Opacity of Liquids. By J. Holker. (With three figures) 216

XXVII. The Relation between the Number and Size of Red Corpuscles and the Opacity of their Suspensions. By J. Holker. (With one figure) 226

XXVIII. The Relation between the Microscopic Appearance of Precipitated Calcium Oxalate and the Opacity of its Suspensions. By J. Holker. (With two figures) 232

XXIX. The Opacity of Serum Diluted with Distilled Water, Physiologically Normal Saline, and Ringer's Solution. By J. Holker. (With two figures) 238

XXX. On the Composition of the Unsaponifiable Matter of the Ether Extract of Human Faeces. By J. A. Gardner 244

XXXI. The Antiscorbutic and Antiberiberi Properties of Certain Sun-dried Vegetables. By J. A. Shorten and C. B. Ray. (With seven figures) 274

XXXII. On an Autoxidisable Constituent of the Cell. By F. G. Hopkins 286

XXXIII. The Influence of the Nervous System on the Excretion of Creatinine. Experiments on Nervous and Mental Patients. By A. A. Weinberg 306

XXXIV. The Salt Effect in Alcoholic Fermentation. By A. Harden and F. R. Henley. (With two figures) 312

PAGE

XXXV. Observations on Abnormal Iodine Values, with special reference to the Sterols and Resins. By I. Smedley MacLean and E. M. Thomas 319

XXXVI. The Effect of Exposure to Low Temperatures on some Physiological, Chemical and Physical Properties of Amphibian Muscle. By D. L. Foster and D. M. Moyle. (With four figures) 334

No. 3

XXXVII. The Influence of Free Fatty Acids in the Intestinal Contents on the Excretion of Calcium and Phosphorus. By S. V. Telfer . . 347

XXXVIII. The Effects of an Unbalanced Diet in the Production of Guinea-pig Scurvy. By G. M. Findlay 355

XXXIX. Note on the Oxygen Consumption of Amphibian Muscle and Nerve. By N. K. Adam. (With one figure) 358

XL. A Critical Study of the Methods of Estimating Cholesterol and Allied Substances. By J. A. Gardner and M. Williams 363

XLI. Note on a Source of Error in the Colorimetric Methods for the Estimation of Cholesterol in Tissue Fats. By J. A. Gardner and F. W. Fox 376

XLII. The Specific Electrical Resistance of Frog's Muscle. By W. Hartree and A. V. Hill. (With one figure) 379

XLIII. On the Stability of Tryptophan in Baryta Hydrolysis. By H. Onslow 383

XLIV. On the Nature of the Substances precipitated by Mercuric Sulphate from Hydrolysed Caseinogen, with reference to the Estimation and Isolation of Tryptophan. By H. Onslow 392

XLV. A Method for the Colorimetric Estimation of Carnosine. By W. M. Clifford 400

XLVI. On the Deterioration of Cotton on Wet Storage. By N. Fleming and A. C. Thaysen. (With Plate I) 407

XLVII. The Nature of the Reducing Substance in Human Blood. By E. A. Cooper and H. Walker 415

XLVIII. The Differential Dialysis of the Antineuritic and the Anti-scorbutic Factors. By S. S. Zilva and M. Miura. (With two figures) . 422

XLIX. The Relation of the Fat-Soluble Factor to Rickets and Growth in Pigs. By S. S. Zilva, J. Golding, J. C. Drummond and K. H. Coward. (With Plate II and three figures) 427

L. The Synthesis of Vitamin B by Yeasts (Preliminary Note). By A. Harden and S. S. Zilva 438

LI. The Action of Salts and Non-Electrolytes upon Buffer Solutions and Amphoteric Electrolytes and the Relation of these Effects to the Permeability of the Cell. By D. Haynes. (With six figures) . . . 440

No. 4

PAGE

LII. Some Observations on the Haemolytic Action of Certain Quinine Salts on the Erythrocytes of Different Individuals and on the Resistance of newly formed Red Corpuscles to Haemolysis under the Influence of Distilled Water. By U. N. Brahmachari and P. Sen 463

LIII. A Method of estimating Phenylhydrazine volumetrically and its Application to the Estimation of Pentosans and Pentoses. By A. R. Ling and D. R. Nanji 466

LIV. On Carrageen (*Chondrus crispus*). II. On the Occurrence of Ethereal Sulphates in the Plant. By P. Haas 469

LV. "Fumed" Oak and Natural Brown Oak. By C. K. Tinkler . 477

LVI. A Method for the Preparation and Recrystallisation of Oxyhaemoglobin. By H. W. Dudley and C. L. Evans 487

LVII. Feeding Experiments in connection with Vitamins A and B: (I) The Value of Steam-distilled Palm Kernel Oil as a Control Fat. (II) Wheat Bran as a Source of Vitamins A and B. By A. D. Stammers. (With two figures) 489

LVIII. The Behaviour of Pectin towards Alkalis and Pectase. By F. Tutin 494

LIX. Further Observations on the Digestion of Fibrin and Caseinogen by Trypsin. By E. S. Edie 498

LX. A Note on the Question of the Identity of Gastric Rennin and Pepsin. By E. S. Edie 507

LXI. The Estimation of Non-Protein Nitrogen in Blood by a Micro-Kjeldahl Method. By E. L. Kennaway. (With one figure) . . . 510

LXII. Denitrification as a Means of Sewage Purification. By E. A. Cooper 513

LXIII. A Method of Protein Removal from Body Fluids for the Purpose of Simultaneous Determination of many Constituents By G. Mukai 516

LXIV. The Antiscorbutic Properties of Concentrated Fruit Juices. Part IV. By A. Harden and R. Robison 521

LXV. Studies on Gelatin. Part I. The Dynamics of the Formation of Gelatin from Ossein. By A. B. Manning and S. B. Schryver. (With four figures) 523

LXVI. The Formation of Vitamin A in Living Plant Tissues. By K. H. Coward and J. C. Drummond. (With three figures) . . . 530

LXVII. Researches on Vitamin A. VII. Notes on the Factors influencing the Value of Milk and Butter as Sources of Vitamin A. By J. C. Drummond, K. H. Coward and A. F. Watson. (With three figures) 540

LXVIII. On the Theory of Gels. By S. C. Bradford. (With Plate III) 553

No. 5

PAGE

LXIX. Excess Protein and Mammary Secretion. By G. A. Hartwell. (With four figures) 563

LXX. Note on the Limitations of the modified Lewis-Benedict method of Blood Sugar Estimation. By R. A. Guy 575

LXXI. The Combinations of Haemoglobin with Oxygen and Carbon Monoxide, and the effects of Acid and Carbon Dioxide. By A. V. Hill. (With one figure) 577

LXXII. The Bactericidal Action of the Quinones and Allied Compounds. By G. T. Morgan and E. A. Cooper 587

LXXIII. Experimental Studies on Cellular Multiplication. I. The Multiplication of Isolated Infusoria. By T. B. Robertson. (With two figures) 595

LXXIV. Experimental Studies on Cellular Multiplication. II. The Influence of Mutual Contiguity upon reproductive rate and the part played therein by the "X-Substance" in Bacterised Infusions which stimulates the Multiplication of Infusoria. By T. B. Robertson 612

LXXV. The Penetration of Electrolytes into Gels. III. The Influence of the Concentration of the Gel on the Coefficient of Diffusion of Sodium Chloride. By W. Stiles and G. S. Adair 620

LXXVI. The Penetration of Electrolytes into Gels. IV. The Diffusion of Sulphates in Gels. By W. Stiles 629

LXXVII. A method for the separation of Amino-acids from the products of Hydrolysis of Proteins and other sources. (Preliminary Communication.) By H. W. Buston and S. B. Schryver 636

LXXVIII. The Pectic Substances of Plants. Part II. A Preliminary Investigation of the Chemistry of the Cell-walls of Plants. By D. H. F. Clayson, F. W. Norris and S. B. Schryver 643

LXXIX. The Quantitative Estimation of the Fat-Soluble Factor. By S. S. Zilva and M. Miura. (With three figures) 654

LXXX. Day and Night Urine during complete rest, Laboratory Routine, Light Muscular Work and Oxygen Administration. By J. A. Campbell and T. A. Webster 660

No. 6

LXXXI. Note on the Fermentation of *i*-Inositol. By J. A. Hewitt and D. B. Steabben 665

LXXXII. The Metabolism of Carbohydrates. Part II. On the possible occurrence of Stereochemical Changes in Equilibrated Solutions of Reducing Sugars introduced into the Circulation. By J. A. Hewitt and D. H. de Souza 667

PAGE

LXXXIII. A Contribution to the Study of the Interconversion of Carbohydrate and Lactic Acid in Muscle. By D. L. Foster and D. M. Moyle. (With one figure) 672

LXXXIV. Blood Enzymes. I. On the Occurrence of Maltase in Mammalian Blood. By A. Compton 681

LXXXV. Urochrome as a Derivative of Chlorophyll. By H. E. Roaf 687

LXXXVI. The Estimation of Carnosine in Muscle Extract. (Preliminary Note.) By G. Hunter 689

LXXXVII. A Study of the Combined Action of Raw Cow's Milk and Orange-juice as Antiscorbutic Substances. By S. Wright. (With two figures) 695

LXXXVIII. The Origin of Milk Fat and its Relation to the Metabolism of Phosphorus. By E. J. Sheehy. (With one figure) 703

LXXXIX. Studies of the Coagulation of the Blood. Part I. Some Physico-Chemical Aspects of Coagulation. By J. W. Pickering and J. A. Hewitt 710

XC. The Distribution of Carnosine in the Animal Kingdom. By W. M. Clifford 725

XCI. A Note on the Physiology of the Ship-worm (*Teredo norvegica*). By C. R. Harington. (With one figure) 736

Index 742

OBITUARY NOTICE

PERCIVAL SPENCER UMFREVILLE PICKERING

CHEMISTS and biochemists everywhere will learn with deep regret of the death of SPENCER PICKERING on the night of Sunday, December 5th, after a long and wearisome illness at the age of 62.

He came of a long line of distinguished ancestors; his father was Percival A. Pickering, Q.C., and his mother was a Stanhope, daughter of John Spencer Stanhope and granddaughter of the famous Coke of Norfolk, Earl of Leicester, by his romantic marriage with his young ward. Pickering was proud of his descent, as well he might be, and spent no little time in editing the family records and tracing their extensive relationships.

He was educated at Eton, but the classics did not appeal to him: when he entered Balliol he read science and in 1881 took a First Class Honours degree. From the outset, however, he was a very independent minded student, and one of his first published papers was a controversion of an opinion held by his tutor.

Being possessed of sufficient private means the question of a career did not worry him as it does some; he was able to follow his bent and pursue his scientific studies unhampered by financial considerations. But he was not attracted by the life at a university. He went down after he had taken his degree and set up a laboratory at his house in Bryanstone Square, London, where he carried out his well-known measurements on the physical properties of mixtures of acids and water.

His results led him to controvert the dissociation hypothesis of Arrhenius. An account of the phenomena as he saw them is given in Thorpe's *Dictionary of Chemistry* under the heading "Solutions." In reading his papers, even those who do not agree with his conclusions are forced to recognise that he handled his data well and was seriously trying to find out what really lay behind them.

Although an independent worker he did not cut himself off from institutions; he accepted a lectureship at Bedford College which he held from 1881 to 1887. He also took part in other intellectual activities and early became a member of the Athenaeum. Had he continued in his work he would have become a useful critic of physical chemistry, standing outside the dissociation school, and he could not have failed to exercise an important influence on the development of the subject.

By a serious accident in the laboratory, however, he had lost an eye and suffered a severe shock which led to so much illness that his doctors advised him to spend part of his time in the country. He accordingly came to Harpenden which though then only a village was already a place of scientific associations, there being no fewer than four Fellows of the Royal Society among its inhabitants—J. B. Lawes, J. H. Gilbert, R. Warington, and

Richard Lydekker. In 1890 Pickering was himself elected into the Royal Society, thus making the fifth. Although not robust in body he was too vigorous in spirit to be idle and he worked on the land. He still continued his physical work for a time but finally he turned his scientific activities in a wholly different direction: he planned out a series of experiments on fruit trees that should do for them what the Rothamsted experiments had done for farm crops. With the aid of the Duke of Bedford, an old college friend, this was accomplished: a field at Woburn was prepared in 1894, planted with fruit trees and the well-known experiments were begun. Seventeen reports have been issued and one, the last, is now in the press. The work has been of considerable interest to biochemists and plant physiologists because of the many problems it raises: it has been of practical importance because it has shown fruit growers that many of their traditional practices require serious overhauling. Pickering delighted in a result that was entirely opposed to conventional doctrine and greatly enjoyed the sceptical amazement with which fruit growers regarded his trees, planted as they thought entirely wrongly, manured entirely wrongly, and yet undeniably growing better and producing more fruit than those managed on ordinary horticultural lines.

It must be admitted that Pickering had good grounds for his attitude, as horticulture in the nineties, however advanced as an art, was in a deplorably backward state as a science.

His first striking result was that the manuring of fruit trees does not conduce to fruit production. This has since been confirmed; it is known that fruitfulness is in some sense opposed to vegetative growth. The result, however, caused some surprise at the time and it was opposed to the doctrine of some of the pundits of the day.

Another surprising result which is not yet accepted by practical growers is that in planting trees the essential thing is to ram tightly, injury to the root during the process being immaterial. The accepted custom is to handle the roots very carefully, to press the soil firmly round, but not to ram. Pickering's view was that intimate contact with the soil stimulated the production of new vigorous roots so that the tree was much better off than if it had been planted in the usual way.

He also studied the effects of fungicides and insecticides and put some of the rather crude formulae on to a sound chemical basis.

Perhaps his most important work from the physiological point of view was concerned with the mutual effects of one growing crop on another. This arose out of an observation at Woburn that the effect of growing grass round apple trees is to arrest all healthy growth and absolutely stunt the tree. The leaves become unhealthy and light coloured, the bark also becomes light coloured, while the fruit loses its green matter and becomes waxy yellow or brilliant red. Where the grassing was done gradually the trees accommodated themselves somewhat to the altered conditions, but never grew so well as when grass was absent.

The effect might have been due to various causes: changes in aeration, temperature, water supply, food supply or physical condition of the soil, but careful experiments failed to show that any of these factors came into play. Covering the soil with cement excluded air at least as thoroughly as grass, and yet did not produce the grass effect, nor was it suppressed by wet seasons, liberal watering, or a supply (in pot experiments) of sufficient water or nutrient solution to keep the soils of grassed and ungrassed trees equally moist, or equally well supplied with food. On the other hand, the grass effect was produced when perforated trays of sand containing growing grass were placed on the surface of the soil in which trees were growing, so that the washings from the grass went straight down to the tree roots. There seemed no possibility of the grass roots in the tray abstracting anything from the soil, and the only explanation appears to be that a toxin is excreted by the grass. Such a toxin however must be very readily decomposed because no toxic properties could be discovered by laboratory tests either in soil that had been removed from the grass roots or in the washings from the above-mentioned trays. He subsequently showed that the phenomena are general and hold whatever crops are grown in pots or trays. In consequence we must be prepared to consider possible toxic effects of one plant on another growing alongside of it, and the parts such effects may play in determining natural plant associations and in explaining some of the bad effects of weeds.

This work will, it is hoped, be developed; it is far too important to remain as it is. It is thoroughly typical of his method: he would make some simple observation that many others must have had the opportunity of making and yet had missed, and then he would show that its significance was far deeper than at first appeared.

This originality of mind combined with accuracy of experimental work and refusal to be bound by any tradition was thoroughly characteristic of the man. His interests, however, were not confined to his investigations. He had clear cut artistic perceptions and surrounded himself in his home with things of beauty. He sympathised much with the leanings of his brother-in-law, William De Morgan, towards the pre-Raphaelite school and he had some of De Morgan's work in his house; its sincerity and fidelity to nature appealed to him, and, moreover, though austere and of iron self-control, he had a strong vein of sentiment in his character. This combination of artistic feeling with high scientific ability is fortunately not rare in England, and when it occurs it is usually associated with some remarkable traits of character. Pickering was a good example of the scholar and gentleman, a welcome member of some of the best circles, and withal one who judged men exactly as he found them, refusing to be bound by convention in the ordinary affairs of life just as he refused its bondage in his scientific work.

E. J. Russell.

I. THE ACTION OF SODIUM HYDROXIDE UPON COAGULATION OF FIBRINOGEN.

By JOHN OGLETHORPE WAKELIN BARRATT.

Beit Memorial Research Fellow.

From the Lister Institute.

(*Received October 28th, 1920.*)

The well-known action of sodium hydroxide in retarding coagulation of fibrinogen in presence of thrombin has been variously interpreted. J. Mellanby [1909] regards this retardation as due to the solution of the newly formed fibrin by the alkali and to a destruction of the ferment. According to W. H. Howell [1916] the effect of addition of alkali is to prevent the formation of fibrin fibrils, though the formation of fibrin aggregates, to which this author regards gelatinisation of fibrinogen as due, may still occur.

With a view of further investigating the action of alkali upon the coagulation of liquids containing fibrinogen and thrombin the present work was undertaken.

It is obvious that if the action of alkali is exerted exclusively upon fibrinogen or is directed solely to thrombin, it would be easy to determine the nature of this action by a comparison of (1) the effect upon coagulation of adding increasing amounts of alkali to plasma or fibrinogen solution with (2) the course of coagulation in a series of dilutions of (*a*) fibrinogen, to each of which the same amount of thrombin has been added, and (*b*) thrombin, to each of which the same amount of plasma has been added.

The effect of dilution of fibrinogen is shown in Table I, that of dilution of thrombin in Table II and the action of alkali in Table III. In these experiments fibrinogen, which cannot at present be prepared in even an approximately pure condition, was represented by citrated human plasma: it is well known that when fibrinogen is precipitated from plasma by dilution and acidification relatively considerable amounts of albumin and globulin are carried down in the precipitate [J. Mellanby, 1905]. As source of thrombin the venom of *Echis carinatus* [Barratt, 1913], dissolved in citrated 0·85 % sodium chloride solution, was employed[1]. The volume of citrated plasma used (undiluted or diluted) was in each experiment 0·050 cc.: to this was added 0·0026 cc. of citrated sodium hydroxide solution of suitable concentration (Table III) or of citrated 0·85 % sodium chloride solution (Tables I

[1] For a supply of this venom I am indebted to the kindness of Dr C. J. Martin, Director of the Lister Institute.

Table I.

To citrated human plasma, undiluted and in the dilutions given below, venom was added in amount required to produce a dilution of 1 in 3,000,000. The time of first appearance of fibrils is calculated from the equation on p. 6.

Plasma %	Time of first appearance of fibrils (min.) observed	calculated	Dimensions (approximate) and aspect of fibrils
100	24	24	·25μ thick; 50μ long; numerous; meshes wide
90	24	24	
80	23	24	·25μ thick; 50μ long; numerous; meshes wide
70	50	55	
60	110	99	·25μ thick; 50μ long; numerous; meshes wide
50	140	151	·25μ thick; 50μ long; less numerous; meshes fairly wide
40	191	215	·2μ thick; 40μ long; less numerous; meshes less wide
30	360	297	·1μ thick; 7μ long; scanty; barely visible
20	no fibrils seen	414	

and II); finally 0·0026 cc. of citrated venom was added, of such concentration as would furnish, after admixture, the titre indicated in the tables. Immediately after mixing, a few cubic millimetres were placed on a microscope slide within an area ringed with vaseline and examined on a dark ground, illuminated by an arc light. The temperature of experiment was 16–18°.

On comparing Tables I and II it will be observed that the effect of dilution of fibrinogen (the amount of thrombin added being the same in each experiment) or of dilution of thrombin (the amount of fibrinogen present being the same in each experiment) is to cause (1) delay in the appearance of fibrin fibrils and (2) diminution in their number. Here, however, the resemblance

Table II.

To citrated human plasma (undiluted) venom was added in amount required to produce the dilutions given below. The times of first appearance of fibrils are calculated on the assumption that concentration of venom × time of first appearance of fibrils = $8{\cdot}3 \times 10^{-6}$.

Venom	Time of first appearance of fibrils (min.) observed	calculated	Dimensions (approximate) and aspect of fibrils
1 : 600,000	5	5	·11μ thick; 5μ long; fine; very numerous; meshes fine
1 : 1,000,000	8	8	
1 : 1,300,000	11·5	11	·2μ thick; 15μ long; numerous; meshes less fine
1 : 1,500,000	10·5	12·5	
1 : 2,000,000	19	16·5	·25μ thick; 40μ long; numerous; meshes wide
1 : 3,000,000	26	25	·25μ thick; 50μ long; numerous; meshes wide
1 : 6,000,000	47	50	·3μ thick; 60μ long; fairly numerous; meshes larger
1 : 12,000,000	175	100	·35μ thick; 80μ long; less numerous; meshes very wide
1 : 24,000,000	350	200	·5μ thick; 150μ long; scanty; coarse

in the two cases ceases: with increasing dilution of fibrinogen the fibrils become short and fine, and ultimately are barely visible (Table I); with increasing dilution of thrombin, on the other hand, the fibrils become long

and coarse (Table II). The effect of adding alkali (Table III) is seen to be identical with that of diluting fibrinogen: this is very strikingly seen when the penultimate experiment in Table III is compared with the penultimate experiment in Table I and with the last experiment in Table II. It follows, therefore, that the action of alkali is exerted upon fibrinogen: no recognisable effect upon thrombin is observable.

The experiments in Table I, when plotted with concentration of plasma as ordinates and time of first appearance of fibrin fibrils as abscissae are found to lie at first upon a vertical line and then upon a curve, as is shown in Fig. 1. Fibrinogen in solution has been shown to be diphasic [Barratt, 1920], the time of first appearance of visible fibrils (made up almost entirely of the period occupied by the growth of the primitive fibrils, which thereby pass from invisibility into the range of ultramicroscopic vision, this growth occurring at the expense of fibrinogen derived from the continuous or dilute phase) being unaltered by dilution until the disperse (concentrated) phase has nearly completely passed into solution (dilute phase), after which further dilution causes a lowering of the concentration of the dilute phase and as a

Table III.

To citrated human plasma (undiluted) sodium hydroxide was added in quantities corresponding to the concentrations given below. Venom was then added in amount required to produce a dilution of 1 in 3,000,000.

Exp.	NaOH	Fibrils first seen at end of	Dimensions (approximate) and aspect of fibrils
1	0	24 min.	·25μ thick; 50μ long; numerous; meshes wide
2	1/120 N	23 „	
3	1·3/120 N	23 „	·2μ thick; 20μ long; numerous; meshes small
4	1·6/120 N	24 „	
5	1·9/120 N	50 „	·2μ thick; 20μ long; very numerous; meshes very small
6	2·2/120 N	54 „	
7	2·5/120 N	63 „	·15μ thick; 10μ long; very numerous; meshes very small
8	2·8/120 N	150 „	
9	3·1/120 N	173 „	·1μ thick; 5μ long; very numerous; barely visible
10	3·4/120 N	285 „	
11	3·7/120 N	345 „	·1μ thick; 5μ long; less numerous; barely visible
12	4/120 N	no fibrils seen	

result retardation of the growth of primitive fibrils (exhibited by a lengthening of the period of first appearance of visible fibrils). The curve, it may be observed, can be calculated from the equation

$$-lc = kt + a$$

where c = concentration of fibrinogen, $t + 24$ = time of first appearance of fibrils and k and a are constants, represented by 0·0035 and − 4·357 respectively.

On reference to Tables I and III it will be seen that in the earlier experiments of each series the period in which visible fibrils first appear is the same,

namely 23 to 24 minutes: only in the later experiments is a retardation observable. This behaviour indicates that the result of adding alkali to plasma is to cause disappearance of fibrinogen, the disperse (concentrated) phase in consequence becoming reduced in amount but the continuous (dilute) phase not being affected until the former phase has nearly completely disappeared, and is therefore no longer able on further dilution to maintain the saturation concentration of the continuous phase, the process occurring being in this respect comparable in its effect to simple dilution of fibrinogen. The concentration of fibrinogen corresponding to the periods given in Table III,

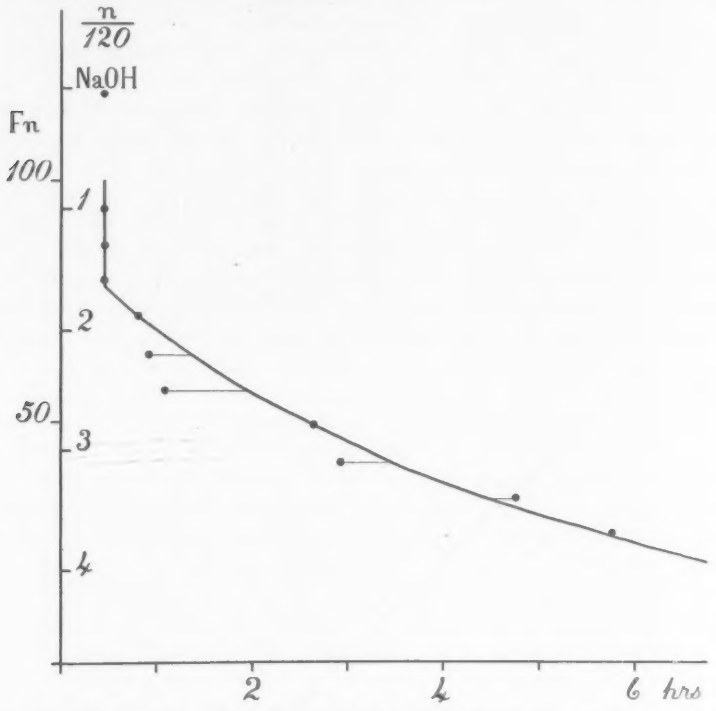


Fig. 1. Graphic record of experiments given in Tables I and III. Ordinates indicate (1) on the left concentration of fibrinogen, that present in undiluted citrated human plasma being represented by 100, and (2) on the right titre of sodium hydroxide; abscissae show time of first appearance of fibrin fibrils. On the curve, which represents the experiments in Table I, the estimated disappearance of fibrinogen on addition of alkali in Tables III and IV is exhibited.

col. 3, may be determined graphically from the curve in Fig. 1 (or by calculation with the aid of the equation on p. 6). The figures so obtained are shown in Table IV, col. 2. If the average amount of fibrinogen disappearing for each increment of alkali is determined (namely that contained in approxi-

mately 7·5 % of plasma for each increment represented by 0·3/120 *N* NaOH) the percentages of fibrinogen given in the third column of Table IV are obtained: these are indicated in Fig. 2, the amount of fibrinogen present in undiluted plasma being represented by 100. (It will be seen from Fig. 2 that no action of alkali on fibrinogen is observable until an amount represented by 0·74/120 *N* has been added.)

Table IV.

Disappearance of fibrinogen on addition of sodium hydroxide in experiments recorded in Table III.

	Fibrinogen	
Exp.	observed	calculated
1	100	100
2	<100	94·5
3	<100	87
4	<100	79·5
5	72	72
6	71	64·5
7	68	57
8	50	49·5
9	47	42
10	32	34·5
11	27	27
12	—	20

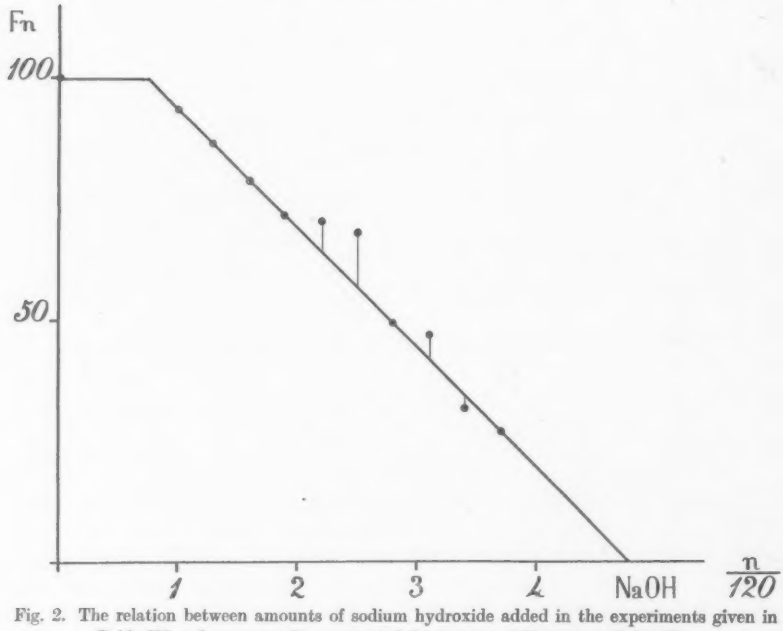


Fig. 2. The relation between amounts of sodium hydroxide added in the experiments given in Table III and corresponding estimated diminution of fibrinogen (Table IV).

The variations from a simple proportionality between addition of alkali and disappearance of fibrinogen in the individual experiments recorded in Tables III and IV are most conveniently exhibited graphically, as in Fig. 2. These variations lie within the error of experiment inseparable from the circumstance that on the one hand the volume in which the thrombin and alkali added is contained must be kept very small relatively to that of the plasma (fibrinogen) employed, in order that the concentration of the latter may not be lowered thereby in any marked degree (in these experiments the respective volumes of (1) plasma and (2) thrombin or alkali solutions employed were 19 : 1), while on the other hand owing to the necessity of rapid working the amounts of fluid used must be small. Although the fibrinogen used for experiment was contained in plasma and the experiments given in Tables III and IV indicate, as already noted, the occurrence of a subsidiary reaction, nevertheless the results obtained suggest that the relation between alkali and fibrinogen is stoichiometric. As is well known fibrinogen is not destroyed by alkali: on addition of carbonic acid to alkali plasma the fibrinogen which has disappeared reappears and clotting in presence of thrombin is now readily obtained. The action of fibrinogen in respect of alkali thus appears to be comparable to that of an extremely weak organic acid.

With the increasing dilution of fibrinogen exhibited in Table I, the fibrin fibrils become gradually finer until ultimately they can no longer be recognised. When this stage is reached there is, however, still evidence of the existence of fibrils, though no longer capable of demonstration by ultramicroscopic illumination, for soft clot continues to be formed *in vitro*. It must therefore be inferred that in such apparently structureless clot amicroscopic fibrils are present, though, owing to the dilution of the continuous phase, no longer able to increase sufficiently in size, by further addition of fibrinogen from this phase, to become visible. In support of this inference, reference may be made to the circumstance that under suitable conditions of experiment the formation of structureless clot precedes visible fibril formation: thus when the dilution of fibrinogen is just insufficient to lead to the non-appearance of fibrin fibrils, "setting" *in vitro* may be observed to precede the appearance of visible fibrils on the microscope slide. It has already been shown [Barratt, 1920] that the formation of primitive (amicroscopic) fibrils precedes by a relatively considerable time the appearance of visible fibrils.

When alkali is added to citrated human plasma the same phenomenon is observable with much greater facility, owing to the circumstance that the volume of solution of alkali added may be made very small compared with that of the plasma employed, and thus the diminution in number of fibrils dependent upon simple dilution avoided, with the result that greater firmness of clot is secured. If sufficient alkali is added to plasma to cause disappearance of visible fibrils, a structureless gel of soft consistence is still obtainable: as more alkali is added gel formation becomes uncertain and ultimately ceases. Thus in a series of experiments of the type shown in Table III, in which the

concentration of venom was 1 in 600,000, no visible fibrils were observed after the fifth experiment: in experiments 6 to 9 "setting" *in vitro* occurred in periods ranging from 30 minutes to 3 hours; in the remaining experiments "setting" was imperfect or did not take place.

The appearance of structureless gel after addition of alkali was noted by Howell [1916], who moreover obtained structureless gel from plasma on dilution and on drying.

Addition of alkali to plasma in amount sufficient to delay fibril formation does not at first affect phenolphthalein. No marked red coloration could be observed until fibrils were no longer recognisable and gel formation absent: in other words, until the amount of sodium hydroxide added was as much as would, if added to a volume of distilled water equal to that of the fibrinogen solution employed, give to the latter a concentration of alkali represented by 1/30 *N*.

Summary.

(1) Sodium hydroxide, added in increasing amounts to citrated plasma, causes the gradual disappearance of fibrinogen from the plasma: the fibrin fibrils observable in the gel forming on addition of thrombin diminish in size and number, and ultimately pass out of the range of ultramicroscopic visibility; when this stage is reached gel formation may be still observable, but the gel obtained is structureless under microscopic illumination, being made up of amicroscopic fibrils; with still further disappearance of fibrinogen gel formation ceases.

(2) No evidence of any action of alkali upon thrombin was obtained.

REFERENCES.

Barratt (1913). *Proc. Roy. Soc.* B. **87**, 179.
—— (1920). *Biochem. J.* **14**, 189.
Howell (1916). *Amer. J. Physiol.* **40**, 526.
Mellanby (1905). *J. Physiol.* **33**, 338.
—— (1909). *J. Physiol.* **38**, 28.

II. THE PRODUCTION OF CARBON MONOXIDE BY THE ACTION OF ALKALINE HYPOHALOGENITES ON UREA.

By WILLIAM HOLDSWORTH HURTLEY.

Chemical Laboratory, St Bartholomew's Hospital.

(*Received November 15th, 1920.*)

R. G. Canti [1916] has used the hypobromite method for obtaining approximate values for the amount of urea in the cerebro-spinal fluid of patients suffering from a variety of diseases. Being desirous of controlling this method by others more reliable he asked R. L. M. Wallis to determine the urea by the urease method in some cases, and G. Graham by the Folin method in others, and the writer to determine the total nitrogen by the Kjeldahl method in other cases.

Here are some typical results:

No. of case	Disease	% urea by NaOBr	% urea by Urease	% urea by Folin	Total nitrogen calculated to urea
48	Uraemia	0·88	0·63	—	—
114	„	0·41	0·10	—	—
115	Carcinoma	0·28	0·18	—	—
75	Uraemia	0·41	—	0·32	—
76	„	0·45	—	0·32	—
97	Chronic nephritis	0·02	—	0·04	—
134	Uraemia	0·75	—	—	2·05
137	„	0·77	—	—	0·75
141	„	0·42	—	—	0·54
157	„	0·45	—	—	0·94

From the table it appears that the volume of gas evolved by acting upon cerebro-spinal fluid with hypobromite, particularly in cases of uraemia, far exceeds the nitrogen contained in the urea of the fluid, but does not exceed the total nitrogen of the fluid. A similar phenomenon is well known in the determination of urea in urine by the hypobromite method. K. H. Mörner [1903] has shown that the nitrogen evolved from urine by the action of hypobromite upon it may exceed the urea nitrogen by more than four times.

Canti and the writer then sought for a constituent other than nitrogen in the gas evolved from cerebro-spinal fluid and hypobromite, a gas which must be practically insoluble in water, and in a solution of sodium hydroxide. Oxygen was shown to be absent. In Canti's case 134 a portion of the gas was shaken up with part of a dilute solution of blood, the other part being kept as a control. To our surprise we obtained the characteristic colour of

carboxyhaemoglobin. On adding yellow ammonium sulphide to one portion of the COHb solution it was not reduced while the same reagent reduced a similar quantity of the diluted blood in the usual way. Another part of the COHb solution was not reduced by sodium hyposulphite while a similar amount of the control solution was instantly reduced. Precisely similar results were obtained in Canti's case 141. These two were cases of uraemia, but the same result was obtained in a case of diabetes and in a case of cerebral softening. These results caused us to test the nitrogen obtained from urea by the action of hypobromite upon it for carbon monoxide, and we found that the latter gas is always present in the nitrogen when hypobromite of the ordinary strength acts upon urea. To be quite certain that the sodium hydroxide used, namely sodium hydroxide "pure by alcohol," had no effect on the result a quantity was prepared direct from sodium, and hypobromite made with it gave with urea nitrogen containing carbon monoxide.

Marie Krogh [1913] had shown that the gas liberated from urea by hypobromite contained carbon monoxide; but when Canti and I discovered it in 1916 we did not know of her paper. By acting upon 1 cc. of a solution of urea containing 9·19 mg. N with hypobromites of different strengths Krogh once more showed that the volume of gas evolved varied with the strength of the hypobromite and she made the important observations (*a*) that the nitrogen contained carbon monoxide; (*b*) that the amount of the carbon monoxide increased with the alkalinity of the hypobromite. For example 1 cc. of the urea solution acted on by 15 cc. of a hypobromite containing 0·193 g.-mol NaOH and 0·0416 g.-atom Br (4·625 : 1) gave total gas 96·2 % of the total nitrogen in the urea and it contained 0·3 % CO; while the same amount of urea with hypobromite containing 0·747 g.-mol NaOH and 0·0203 g.-atom Br (36·86 : 1) gave total gas 99·4 % of the total nitrogen and it contained 0·62 % CO. Krogh was led to suspect the presence of carbon monoxide in the nitrogen by the fact that when she acted upon 1 cc. of urea of the afore-mentioned strength and 1 cc. of a 25 % solution of glucose with hypobromite of the concentrations just given the total gas evolved was 101·1 and 101·7 % respectively of the urea nitrogen. This being the case the gas from urea and that from urea together with glucose was analysed in an apparatus which appears to have been similar to that of Haldane; and after combustion a contraction was observed which was half that obtained on submitting the product to the action of sodium hydroxide. Krogh therefore concluded that the gas must contain a volume of carbon monoxide equal to the contraction after treatment with sodium hydroxide. No qualitative tests such as the blood test are described, nor is it stated that oxygen was added to burn the carbon monoxide. If the oxygen required for combustion was supplied by the air of the generating vessel then the results would be low. Canti and I estimated the carbon monoxide in the gas from the cerebro-spinal fluid of case 142 and in that from a solution of pure urea. To collect the gas without mixing it with air a Lunge nitrometer was filled with hypobromite

of the usual strength (2–5 cc. Br to 25 cc. of a solution of sodium hydroxide containing 40 g. NaOH in 100 cc.) and the fluid or the urea solution was added from the cup. A small Haldane's apparatus was used for the analysis; a suitable volume of gas having been admitted to the apparatus and measured about 2 cc. oxygen were added, the reading taken and the analysis made.

	Vol. of gas admitted cc.	Contraction on combustion cc.	Contraction after KOH cc.	CO %
Case 142 (x. 1916)	7·16	0·05	0·10	1·4
Pure urea (x. 1916)	7·07	0·025	0·07	0·99

Subsequent determinations of the carbon monoxide by the writer have given a smaller percentage than this early one. In the following determinations about 120 cc. of gas were liberated from urea as just described and of this gas 100 cc. were mixed with 20·4 cc. of oxygen. This mixture was analysed in the Haldane's apparatus:

Vol. of mixture cc.	Contraction on combustion cc.	Contraction after KOH cc.	CO %
9·74	0·05	0·06	0·74
9·68	0·02	0·06	0·75

In the following determinations a flask holding 255 cc. to the bottom of the neck was charged with alkaline hypobromite previously made from 20 cc. bromine and 200 cc. of sodium hydroxide: the flask was fitted with a rubber stopper carrying a small graduated tap funnel and a short delivery tube attached to a nitrometer. To displace the air from the flask and delivery tubes 20 cc. of 2 % urea solution were run into the flask. This gas having been expelled from the nitrometer, more urea was added to the flask and the next 150 cc. of gas collected; 40 cc. of it were mixed with 10 cc. of oxygen and the mixture analysed. To control these results a mixture of air and carbon monoxide containing about 2 % of the latter gas was made and stored over water. Three analyses of this mixture were made; then 25 cc. of the mixture were diluted with 25 cc. of air and two analyses of this were made. The table gives the results of two analyses of the gas from urea, and the analyses of the air containing carbon monoxide.

Vol. of mixture cc.	Contraction on combustion cc.	Contraction after KOH cc.	CO %
9·89	0·04	0·05	0·63
9·96	0·035	0·055	0·69
9·93	0·09	0·16	1·61
9·89	0·07	0·175	1·77
9·92	0·07	0·16	1·61
9·84	0·045	0·075	0·76
9·90	0·040	0·090	0·91

From these results it may be concluded that the nitrogen evolved from a 2 % solution of urea, when acted upon by hypobromite of the usual strength,

contains 0·7 % of carbon monoxide. Since this percentage of carbon monoxide is easily detected by shaking the gas with diluted blood its presence can easily be demonstrated in class or in lecture. For this purpose the gas from 10 cc. of a 2 % solution of urea and 50 cc. of hypobromite generated in the usual bottle, may be collected in a nitrometer. From this apparatus the gas can be driven into a 150 cc. narrow necked measuring cylinder filled with suitably diluted blood and inverted in an evaporating basin containing the same. The gas collected, the stopper is inserted, and the cylinder is well shaken. A 50 cc. Nessler glass is now filled to the mark with blood from the cylinder and a similar glass is filled to the mark with some of the original diluted blood. The difference in colour is very marked, and on the addition of a trace of solid sodium hyposulphite to each glass it will be seen that the COHb remains unchanged while the control is instantly reduced on shaking.

The Origin of the Carbon Monoxide.

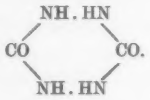
Krogh [1913] showed that, when alkaline hypobromite rich in hypobromite was employed to act upon urea, less nitrogen and also less carbon monoxide were produced than when the alkaline hypobromite was poor in hypobromite and rich in sodium hydroxide (see her figures quoted above). She assumes that in the former case more carbon is oxidised to carbon dioxide and more nitrogen is oxidised to some oxide of nitrogen, which remains combined with base as nitrite or nitrate, than in the latter case and she makes experiments to prove that the residual nitrogen is present as nitrate. Nitrate had been previously found under certain conditions in the residue from this action of hypobromites on urea by Fauconier [1880] and by R. Luther [1889].

It seemed desirable to ascertain whether sodium hypochlorite when it acts upon urea also gives carbon monoxide. H. J. H. Fenton [1878], using sodium hypochlorite whose strength he does not give, obtained according as he used hypochlorite alone or mixed with sodium hydroxide 49·72 % of the nitrogen in urea; but when the same hypochlorite was used with sodium carbonate he obtained 92·51 %. The writer obtained the results embodied in the following table. Washed chlorine was passed into sodium hydroxide (40 g. NaOH in 100 cc.) cooled in ice, until the solution was of such a strength that 1 cc. of the clear liquid was equivalent to 62·8 cc. $N/10$ sodium thiosulphate; the usual bottle and tube was used for the reaction and the gas was collected over water in a Lunge nitrometer.

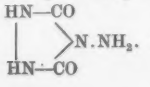
Exp.	Urea 2 g. in 100 cc.	NaOCl solution	NaOH 40 g. in 100 cc.	Na_2CO_3 saturated	NaOCl cc. Normal	% of total *N* in urea
1	5 cc.	25 cc.	—	—	157	61·3
2	„	25	—	25 cc.	157	54·8
3	„	25	25 cc.	—	157	77·8
4	„	10	40	—	62·8	84·5
5	„	5	45	—	31·4	81·6
6	„	2	48	—	12·5	78·2
7	„	10	—	40 cc.	62·8	53·8

Here it is seen that a concentrated alkaline hypochlorite gives much less gas than a hypochlorite of one-fifth the strength but containing far more alkali. Experiment 4 was repeated on a large scale in order to collect a quantity of the gas free from air and to examine it for carbon monoixde. In 50 cc. of the gas carbon monoxide could be detected, but 100 cc. were required to give a really good test. The amount was too small for estimation in my Haldane's apparatus (capacity 10 cc.). It is certain that although carbon monoxide appears over a wide range of concentrations of hypochlorite it is always less in amount than in the gas from hypobromite. Fenton [1878] showed that cyanate was present in the residual liquid from the action of hypochlorite. Nitrate had been shown to be present by Fauconier [1880] and Luther [1889].

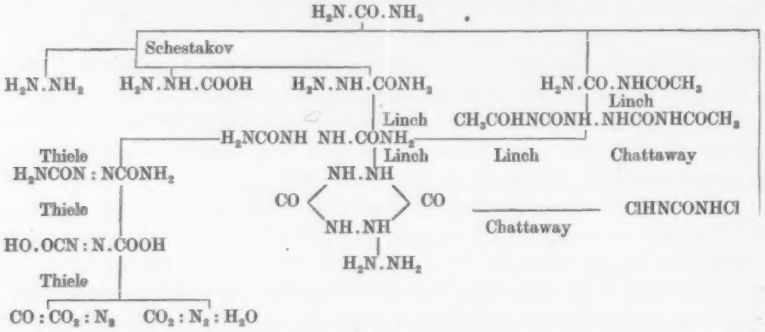
It is certain from what precedes that the action of hypohalogenite upon urea is a complex occurrence. In this paper the question of residual nitrogen is not discussed; only an attempt will be made to explain the formation of carbon monoxide. Schestakov [1905] showed that when an alkaline hypobromite or hypochlorite acts upon urea the following compounds can be isolated from the residue in the form of their benzaldehyde derivatives; hydrazine, semicarbazide, hydrazinecarboxylic acid—the first of these in a yield of 60 %. Chattaway [1909, 1, 2] showed that when chlorine is passed into a strong aqueous solution of urea, or better into such a solution in presence of zinc oxide, a good yield of dichlorocarbamide is obtained and that this compound when acted on by ammonia forms *p*-urazine[1]



This compound on hydrolysis by acids yields salts of hydrazine. Linch [1912] showed that when sodium hypobromite acts upon semicarbazide an 80 % yield of *p*-urazine is obtained, and in this reaction he believes that hydrazodicarbonamide is an intermediate product for he shows that hydrazodicarbonamide itself when acted upon by hypobromite also yields *p*-urazine. He further showed that acetylcarbamide acted upon by hypobromite gave diacetylhydrazodicarbonamide which was partly hydrolysed by the alkaline liquid to hydrazodicarbonamide. Thiele [1892] showed that hydrazodicarbonamide is oxidised by chromic acid to azodicarbonamide and that the latter can be hydrolysed to potassium azodicarboxylate. This salt gives a bright yellow solution in water, its aqueous solution is unstable but is stabilised by alkali, and the salt when heated a little above 100° gives carbon monoxide.

[1] Busch regards the *p*-urazines as urazoles and on his view this compound would be amino-urazole.  I am not convinced that his arguments apply to this compound.

When the salt is decomposed by water alone only nitrogen is evolved. All these reactions are summarised in the following scheme:



In order to trace the origin of the carbon monoxide formed by the action of hypohalogenite upon urea the action of alkaline hypobromite upon many of the substances in the above table has been examined.

Hydrazocarboxylic acid and its hydrazine salt. As recommended by Stolle and Hofmann [1904] carbon dioxide was passed into a well cooled solution of hydrazine hydrate, and a part of the solution was acted on by hypobromite of the usual strength. No carbon monoxide could be detected in the nitrogen evolved. Another part of the solution was evaporated in a vacuum over sulphuric acid and the deliquescent crystals of the hydrazine salt of the acid also failed to give any carbon monoxide with hypobromite.

Dichlorocarbamide. An aqueous solution of this compound was run into strong ammonia and the gas evolved collected in a nitrometer. The yield of gas assumed to be nitrogen was 10·52 % of the dichlorocarbamide used: theory 21·73. But the gas was rich in carbon monoxide, containing as the mean of three concordant results 1·24 % CO. When sodium hydroxide was used in place of ammonia the evolved gas also contained carbon monoxide. In all cases a crystalline precipitate of *p*-urazine was obtained as described by Chattaway.

p-*Urazine.* This compound acted upon by hypobromite of the usual strength also gave monoxide, the amount of which has not yet been determined.

Semicarbazide. Linch's experiment on this compound (1 g.-mol semicarbazide hydrochloride, 4 g.-mols NaOH and 3 g.-atoms Br) was repeated with the modification that the evolved gas which Linch assumes to be nitrogen, was collected and examined. It was found to contain 22 % of carbon dioxide and also some carbon monoxide. The amount of the latter gas was 0·64 %—slightly less than that given by urea under ordinary conditions.

Acetylcarbamide with hypobromite of the usual strength also gave carbon monoxide—amount not yet estimated.

Hydrazodicarbonamide. 0·5 g. of the solid was placed in a dry tube which

was put into the usual bottle containing hypobromite made from 50 cc. of 10 *N* NaOH and 5 cc. Br (1 g.-mol: 119 g.-mols: 46·4 g.-atoms) and the gas collected in a nitrometer. The action was quite slow and it gave 101·45 cc. moist nitrogen at 67° and 770 mm. This is equivalent to 28·23 g. N per g. molecule, that is half the total nitrogen. It contained no trace of carbon monoxide.

Linch's experiment with this compound was repeated (1 g.-mol: 2 g.-mols: 2·33 g.-atoms), much less nitrogen was evolved but now the gas contained a little carbon monoxide. Therefore the following experiment was tried: 0·59 g. of the compound was thoroughly shaken with 50 cc. water in the bottle. Into this tube 10 cc. of 10 *N* NaOH was placed, cooled to 0°, and 0·5 cc. Br added. (1 g.-mol: 20 g.-mols: 3·9 g.-atoms.) On mixing these 66 cc. N_2 at 20° and 764 mm. were obtained = 15·22 g. N per g.-mol or rather more than 1 g. atom. This gas gave a strong reaction for carbon monoxide. Its amount has not been determined. When the hydrazo-dicarbonamide dissolves in the hypobromite it gives a clear and intensely yellow coloured solution.

Azodicarbonamide. The first experiment with this compound was made in exactly the same way as the first experiment with hydrazodicarbonamide and with similar quantities. There is however a great difference in the speed of the reaction which in this case is very high and much heat is evolved. Calculated on the g.-molecule the nitrogen evolved was 38·67 g. or 2·75 g.-atoms. No trace of carbon monoxide could be detected in this gas. This experiment was repeated as in the last experiment with hydrazodicarbonamide using 0·58 g., 5 cc. 10 *N* NaOH, and 0·8 g. Br (1 g.-mol: 10 g.-mols: 1 g.-atom). In this case the yield of nitrogen was 10·68 g. per 1 g.-mol substance, that is 0·75 g.-atom, and the gas gave good tests for carbon monoxide but the amount has not yet been determined. The remaining solution was bright yellow.

Schestakov explains the formation of hydrazine, which he isolated as benzaldazine, and of hydrazocarboxylic acid which he also isolated in small amounts as its benzaldehyde derivative by the following scheme:

$$\underset{\underset{\|}{\mathrm{NH}}}{\mathrm{H_2N.C.OH}} \longrightarrow \underset{\underset{\|}{\mathrm{N.Br}}}{\mathrm{H_2N.C.OH}} \longrightarrow \underset{\underset{\|}{\mathrm{N.NH_2}}}{\mathrm{Br.C.OH}} \longrightarrow \underset{\underset{\|}{\mathrm{N.NH_2}}}{\mathrm{HO.C.OH}} \longrightarrow \mathrm{HOOC.NH.NH_2} \longrightarrow \mathrm{H_2N.NH_2}$$

and he believes that the hydrazocarboxylic acid is the compound which furnishes the nitrogen obtained by the action of hypobromite on urea. This is probably the main series of reactions; but these reactions alone fail to explain (1) the formation of semicarbazide which Schestakov isolated in small amount as benzalsemicarbazide, (2) the production of carbon monoxide. The writer would suggest provisionally that in addition to the reactions indicated by Schestakov the following occur:

$$\mathrm{H_2N.CO.NH_2} \rightleftharpoons \mathrm{HCNO} + \mathrm{NH_3}.$$

That this reaction does occur is made very probable by Fenton's observation

[1878] that cyanate is contained in the residual solution when sodium hypochlorite acts upon urea. Then

$$H_2N.C(OH):NBr + NH_3 = H_2N.CO.NH.NH_2.$$

From the semicarbazide, hydrazodicarbonamide is next produced as indicated by Linch, then azodicarbonamide by oxidation by hypobromite and finally carbon monoxide as outlined in the scheme on p. 16. This work is incomplete, but it is being continued in the hope of further elucidating the changes which occur when hypohalogenites act upon urea.

The writer wishes to record his hearty thanks to Dr R. G. Canti for permission to publish their joint work, and also to Mr J. H. Johnston, M.Sc. and Mr S. D. Barlow for help with many of the determinations and preparations involved in the work.

Summary.

Krogh has shown that carbon monoxide is produced as well as nitrogen when sodium hypobromite acts upon urea whether in urine or in solution in water. Krogh obtained the result by combustion of the carbon monoxide.

The author and Canti showed by the blood test and by combustion that carbon monoxide is also formed when sodium hypobromite acts upon the urea contained in cerebro-spinal fluid and also on pure urea. Its production admits of an easy class or lecture demonstration.

The amount of carbon monoxide contained in the nitrogen when sodium hypobromite of the usual strength is used to act upon a 2 % solution of urea is 0·7 % of the total gas.

Carbon monoxide is also produced along with nitrogen when sodium hypochlorite acts upon urea, when ammonia or caustic soda acts upon dichlorocarbamide, and when sodium hypobromite of suitable strength acts upon semicarbazide, hydrazodicarbonamide, azodicarbonamide, and acetylcarbamide.

References.

Canti (1916). *Lancet*, February 12th.
Chattaway (1909, 1). *J. Chem. Soc.* **95**, 234.
—— (1909, 2). *J. Chem. Soc.* **95**, 464.
Fauconier (1880). *Bull. Soc. Chim.* (2), **3**, 102.
Fenton (1878). *J. Chem. Soc.* **33**, 300.
Krogh (1913). *Zeitsch. physiol. Chem.* **84**, 379.
Linch (1912). *J. Chem. Soc.* **101**, 1755.
Luther (1889). *Zeitsch. physiol. Chem.* **13**, 500.
Mörner (1903). *Skand. Arch. Physiol.* **14**, 321.
Schestakov (1905). *J. Russ. Chem. Soc.* **37**, 1. DRP. 164755. French patent 329430.
Stolle and Hofmann (1904). *Ber.* **37**, 4523.
Thiele (1892). *Annalen*, **271**, 127.

III. THE EFFECT ON KITTENS OF A DIET DEFICIENT IN ANIMAL FAT.

By HELEN MARION MACPHERSON MACKAY.

Beit Memorial Research Fellow.

The Department of Experimental Pathology, Lister Institute.

(*Received November 15th, 1920.*)

Mellanby [1919] in the course of his experiments on dogs has come to the conclusion that rickets is a dietetic disease, and that the ultimate cause is a deficiency in the diet of "anti-rachitic factor" a substance which he thinks is probably identical with fat-soluble *A*. He has said that 100 % of young puppies fed on his basal diets develop rickets.

Since the cat, like the dog and man, subsists on a mixed dietary it was decided to investigate whether kittens fed on a diet similar to Mellanby's basal diet would develop rickets. Before describing the experiments on kittens it is of interest to compare the normal diet of young pups and kittens [Tibbles, 1912]:

	Cat's milk	Bitch's milk	Cow's milk
	%	%	%
Protein	9·12	11·15	3·57
Fat	3·33	9·57	3·69
Sugar	4·91	3·08	4·88
Ash	0·61	0·73	0·71

It will be seen from the foregoing table that the percentage of protein is far higher in the milk of the cat and the bitch than in that of the cow; bitch's milk contains nearly three times the percentage of fat present in cat's milk, and the fat content of cat's milk and cow's milk is roughly the same. If the percentage of fat in these three milks could be taken as an index of the quantity of fat-soluble *A* present it would point to the fact that the puppy requires a large quantity of this in its diet, and would afford an explanation on Mellanby's hypothesis of the apparently great liability of young dogs to rickets as compared with other domestic animals.

The latest of Mellanby's published basal diets consists of the following substances:

Separated milk	...	250–350 cc. daily
White bread...	...	*ad lib.*
Linseed oil ...	...	5–15 cc. „
Yeast ...	...	5–15 g. „
Orange juice...	...	3 cc. „
Sodium chloride	...	1–2 g. „

All the fat-soluble *A* content of milk is not present in the fat [see McCollum, 1917], but no accurate data are at present available from which it is possible to make any trustworthy calculation of the amount of this factor present in the quantity of separated milk (250–350 cc.) given in the foregoing diet.

Experiment 1.

Housing and Diet of Kittens.

The five kittens used in the experiment belonged to three litters. They were housed and fed in two pens (controls in one and experimental animals in the other) in a warmed room. They were allowed out for several hours every day and had abundant exercise. The diet is given in the following table:

Control kittens, Nos. 2 and 3	Experimental kittens, Nos. 1, 4, and 5
Whole milk *ad lib.*	Machine skimmed milk with 3 % olive oil *ad lib.*
White bread *ad lib.*	Same as for controls
Orange juice 1 cc. (hand fed) alternate days ...	
Marmite 2 g. given in milk, whole not consumed	

The milk, whole and skimmed, was brought to the boil and allowed to cool before being given to the kittens—a proceeding which probably destroys only a very small part of its anti-scorbutic value [Barnes and Hume, 1919]. The average fat content of a week's supply of machine skimmed milk estimated by Soxhlet's method was 0·15 %[1]. The olive oil used after the first six weeks was kindly supplied by Dr Zilva from a consignment of autoclaved oil which gave a negative result for fat-soluble *A* in rat experiments (linseed oil had been tried but was refused by the kittens). The oil emulsified fairly well with hot skimmed milk but separated out when allowed to stand, so that the quantity of oil consumed was not necessarily 3 % of the skimmed milk intake. The dose of orange juice was a small one, but it was thought that with a diet consisting chiefly of milk a small dose would ensure against anti-scorbutic deficiency. A larger amount was at first given but appeared to give rise to diarrhoea.

The marmite, which was used in place of the yeast to supply additional water-soluble *B* accessory food factor, was given in the day's supply of milk. It is not possible to give the food intake of each individual kitten as the food (with the exception of the orange juice which was given with a pipette) was left in the pens. But the following table gives the average consumption; the quantity of bread is a very rough approximation as it was impossible to estimate with accuracy the residues left.

[1] On a few occasions dried machine skimmed milk or hand skimmed milk was given on account of the failure of the supply of separated milk from the dairy.

Average daily food consumption of each kitten of experiment 1.

	Control kittens 2 and 3	Experimental kittens 1, 4, and 5
Whole milk	About 200 cc. each	—
Skimmed milk with 3 % olive oil	—	About 145 cc. each
White bread	„ 25 g. „	„ 20 g. „
Orange juice	0·5 cc.	0·5 cc.
Marmite	1–2 g.	1–2 g.

Course of experiment.

The accompanying charts (Figs. 1 and 2) show the weight curves of the kittens. But at this stage it must be mentioned that every one of the five kittens, especially the three experimental animals, was latterly very heavily infested with fleas, and that there were in each case at post mortem vast numbers of tape worms in the small intestines. This tape worm, the *Diplidium caninum*, has for its secondary host the dog or cat flea. To what extent these parasites affected the ultimate result is not clear, but all the kittens suffered from time to time with loose stools, their rest was disturbed by the fleas, and at the end of the experiment neither of the two control animals was in perfect condition; kitten 2 was well nourished and active but had loose stools, No. 3 was very thin. The experimental kittens were from the first more keenly interested in their food than the controls—they appeared to feel more hungry though their consumption of food was on the average smaller. About the sixth week of the experiment it was obvious that the experimental kittens were thinner than control kitten No. 2 which showed a steady gain in weight after the first three days (control No. 3 was however thin also). By the tenth or eleventh week the experimental animals were extremely emaciated, with coats in poor condition and were becoming less active. The emaciation, the condition of the coat and the inactivity became steadily worse and latterly the abdomens were much distended. At the end of the experiment there was an obvious difference in size between the two control kittens and the other two of the same litters. The difference in length from tip of nose to end of tail was 2 ins. and 1 in. respectively.

All the kittens appeared anaemic, possibly partly as a result of the tape worm infection. The blood count for kitten 5 (on the deficient diet) on the 114th day of the experiment is given below:

	Kitten 5	Normal blood count for cat [Gulland and Goodall, 1914]
Red blood corpuscles	4,390,000 per cmm.	8,000,000 per cmm.
Haemoglobin	21 %	70 %
White blood corpuscles	22,300 per cmm.	18,000 per cmm.

One of the skimmed milk animals, kitten 1, died on the 82nd day, the other two, kittens 4 and 5, were killed on the 114th day.

Post mortem findings.

At post mortem no evidence of rickets was discovered. The pathological findings are given below. It will be remembered that the three kittens on the deficient diet were kittens 1, 4 and 5, those on the theoretically complete diet kittens 2 and 3.

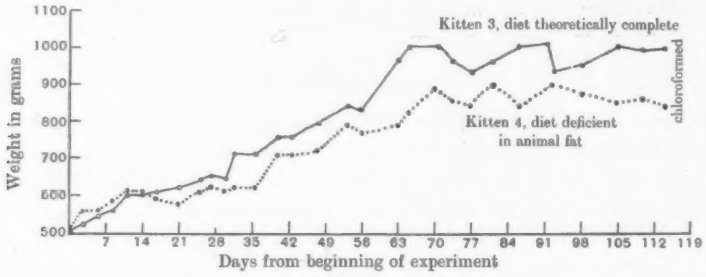


Fig. 1.

Control Kitten, 3 ♀ o——o
Experimental Kitten, 4 ♀ •.....• } Same litter (6 weeks old)

It will be seen that this Control Kitten did not thrive

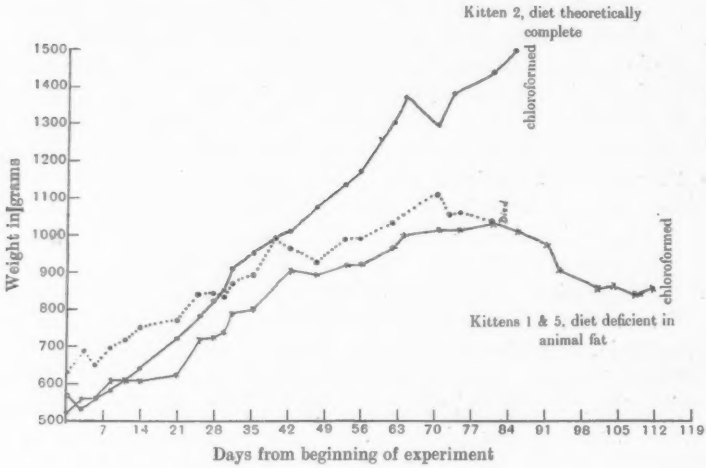


Fig. 2.

Control Kitten, 2 ♀ o——o
Experimental Kitten, 1 ♀ •.....• } Same litter
Experimental Kitten, 5 ♀ ×——×

EXPERIMENTAL KITTENS 4 AND 5. Killed 114th day. Extremely emaciated. *Costo-chondral junctions* slightly enlarged. The histological changes as will be seen from the following paper written by Miss F. M. Tozer [1921] resembled those found in guinea-pigs and rats receiving a diet deficient in fat-soluble *A*.

Stomach and intestines were distended with more or less complete loss of the normal puckering. The walls were much thinner than in the normal animals. The changes were similar to those described by McCarrison [1920] in animals receiving deficient diets. *Liver* pale and fatty looking—that of kitten 5 small compared to the controls. *Kidneys* very pale. *Thymus* absent.

Experimental Kitten 1. Died 82nd day. Extremely emaciated.

Costo-chondral junctions very slightly enlarged [see Tozer, 1921]. *Stomach and intestines* appeared normal. *Liver* pale fatty and small. *Kidneys* appeared normal. *Thymus* absent.

Control Kitten 2. General condition excellent, nothing abnormal discovered excepting the parasites which were found in all the animals.

Control Kitten 3. Very thin. No cause found unless the condition was due to the presence of the parasites.

The teeth of all the kittens were perfect. In the two control animals whose costo-chondral junctions were normal [but see Tozer, 1921] the thymus gland was present, whereas as will be seen above it was absent from the three experimental animals.

Summary. To summarise it appears that the chief effects of the diet deficient in fat-soluble *A* on these three six-week-old kittens were emaciation, arrest of growth, abdominal distention with (in two animals) atrophy of the walls of the stomach and intestines, and changes in the costo-chondral junctions similar to those seen in guinea-pigs and rats suffering from deficiency of the same accessory food factor.

The weak point of the experiment is of course the presence of the intestinal parasites and the fact that one of the two controls did not increase satisfactorily in weight.

Experiment 2.

Children who develop rickets generally do so during the period of the first dentition—a stage of development at which they are, comparatively, much less mature than six-week-old kittens. Therefore while the first experiment was running it was decided to start a second one, this time with younger animals.

Age and diet of kittens. Of a litter of four kittens aged 16 days, three, after certain preliminary "test feeds," were removed from the mother and hand fed entirely until nearly five weeks old, and partially until 7–8 weeks old. They were fed three-hourly from 10 a.m. to 10 p.m. with warmed food from a pipette or baby's bottle until old enough to feed themselves. The food was on the same lines as in the last experiment.

Average daily food consumption of each kitten of experiment 2.

	Control kitten 9	Experimental kitten 6	Experimental kitten 7
Whole milk	About 180 cc.	—	—
Skimmed milk with 3 % olive oil ...	—	About 145 cc.	About 160cc.
White bread supplied *ad lib.* after 3rd week of experiment	A very small quantity	Same as for control	
Orange juice hand fed after 3rd week	0·5 cc.		
Marmite	1–2 g.		

Course of experiment (see Fig. 3).

The two experimental kittens appeared always ravenous, though latterly their food intake was decidedly smaller than that of the control. This was a striking feature almost to the time of death. About the 4th day kitten 7 had diarrhoea, a condition from which both experimental kittens frequently suffered, especially kitten 7 (though both had masses of hard faeces in the large intestine at death). By the 8th day kitten 7 was less active than the control, the abdomen was distended and it walked very unsteadily on a broad base with the back bowed. By the 12th day kitten 6 was in much the same condition. They became progressively worse in all respects, the coat was matted and dirty and swarming with fleas, the hair coming out and there

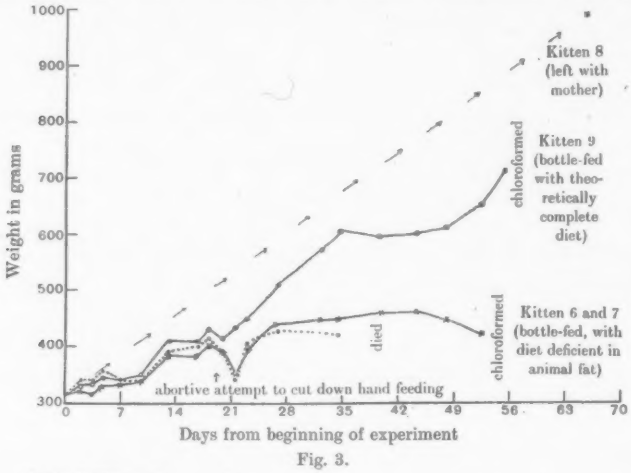


Fig. 3.

Control Kitten, 9 ♀ o——o
Control Kitten, 8. Left with mother, final weight indicated ——→ ×
Experimental Kitten, 6 ♂ ×——×
Experimental Kitten, 7 ♂ •....•

was emaciation and arrest of growth. By the end of the experiment the contrast was striking. Kitten 9, though considerably smaller than the single kitten left with the mother, was a healthy active young cat, whereas the two animals on deficient diets were wasted dwarfs with ballooned abdomens and matted hair. They presented a miserable spectacle. The lengths of the kittens quoted below give no idea of the apparent difference in size. Kitten 7 died on the 38th day with an attack of vomiting, kitten 6 appeared moribund and was chloroformed on the 54th day.

Comparative lengths of kittens (inches)

Kitten	35th day	54th day
9	14·75	17·75
7	12·5	—
6	13·0	14·75

Post mortem findings.

At post mortem it was found that all three kittens had tape worms, the control far more than the two on the deficient diet. The fat and the healthy looking well-developed muscles of control kitten 9 were in marked contrast to the pale flabby wasted tissues of the two small experimental kittens. The two experimental animals had slightly enlarged costo-chondral junctions (for the histological changes, which resembled those found in the earlier experiment, see Tozer [1921]). The rib junctions of the control were normal. The teeth were perfect.

The *stomach and intestines* showed dilatation and atrophy of the wall. The *liver* was pale and small compared to that of kitten 9. The *kidneys* appeared normal. The *thymus* was absent from all three kittens.

Summary. The results of this experiment were similar to those of the last, but the arrest of growth, emaciation, abdominal distention, weakness and the hankering for food were early and very marked features. The picture seems extremely like that presented by children suffering from coeliac disease, a condition in which there is failure to digest fat, arrest of growth, emaciation, distention of the abdomen and hankering for food, as well as, in some cases, a diminution in size of the liver. Still [1918], after remarking on the apathy and langour of children with coeliac disease, observes "There is one subject in which almost all children with coeliac disease show a pathetic interest—namely their food; often indeed it is their one subject of conversation." He however attributes this to the meagre dietary which has formed a part of their treatment.

Criticisms of the experimental procedure.

If these experiments were repeated it would be desirable to feed the kittens separately in order to ascertain the food intake of each, and for the same reason to give the oil by hand instead of as an emulsion in the milk.

A better control would be provided by feeding the whole litter on skimmed milk, and to the control animals giving say cod liver oil, which is rich in fat-soluble *A*, in place of the olive oil of the experimental animals, for it is probable that the mechanical factor introduced by the oil was in part the cause of the diarrhoea in kittens 6 and 7. It might be as well also to give a larger dose of anti-scorbutic, possibly in the form of neutralised orange juice.

As already pointed out the presence of the tape worms and their hosts the fleas introduced an unlooked-for difficulty.

EXPERIMENT 3.

Young rats aged 13 days (the eyes had opened on the 12th day) were put on a diet of white bread, skimmed milk with 3 % olive oil and small pieces of orange pulp. They consumed a much smaller quantity of milk as compared

with bread than did the kittens—but as will be seen from the weight chart (Fig. 4) their weight was not greatly below normal at the end of 122 days—their general condition was excellent. It would appear therefore that kittens require a larger proportion of fat-soluble *A* in their dietary than do young rats.

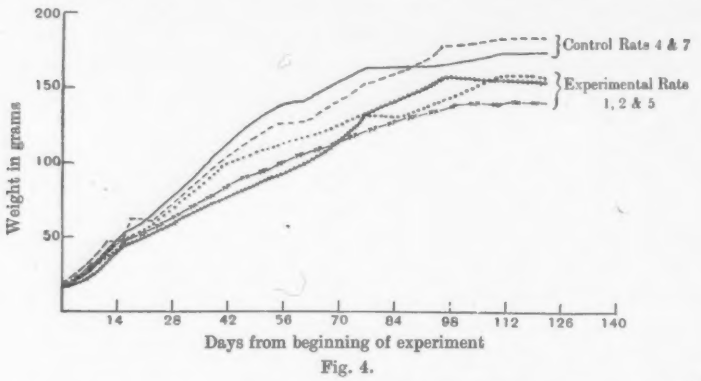


Fig. 4.

Control rat, 4 ♀ ——— Whole milk, bread and orange juice from 13 days old
,, rat, 7 ♀ - - - - Left with mother—mixed diet
Experimental rat, 1 ♀ ××—×× } Skimmed milk with 3 % olive oil, bread and
,, rat, 2 ♀ ||||| } orange juice from 13 days old
,, rat, 5 ♀ ..... }

General Summary and Conclusions.

In the experiments described above it was found that the six-week-old kittens when fed on a diet deficient in fat-soluble *A*, but otherwise theoretically adequate, became emaciated and finally ceased growing. They suffered from abdominal distention and diarrhoea; at post mortem it was found that the walls of the intestine were thin, and there were changes in the costo-chondral junctions resembling those found in young guinea-pigs and rats suffering from deficiency of the same accessory food factor. The thymus was present in two control animals and absent from the three experimental animals, but in the next experiment was absent from control and experimental animals alike. The food of these experimental kittens consisted of skimmed milk, olive oil and bread, with marmite and orange juice. Some weak points in the experiment have been pointed out.

Analogous changes were observed in a second experiment on kittens aged 16 days, which were hand-fed, and given a similar dietary—but in this case all the changes were much more rapid and striking. The appearance presented by these animals was extremely like the clinical picture of coeliac disease in children. There was no evidence of rickets found in any of the kittens post mortem, but the experiments appear to indicate that kittens when given a

diet deficient in fat-soluble *A* develop a condition similar to that found in guinea-pigs and rats under the same circumstances, and that kittens are probably more sensitive than young rats to a partial deficiency of the fat-soluble accessory food factor.

In conclusion I should like to offer my sincere thanks to Dr C. J. Martin for his stimulating assistance and advice. The greater part of the laborious task of feeding the animals was carried out by Miss H. Henderson Smith, and to her as well as to Miss S. Rutherford and others who shared in the work I owe the possibility of carrying out the experiments.

REFERENCES.

Barnes and Hume (1919). *Lancet*, ii, Aug. 23rd.

Gulland and Goodall (1914). *The Blood: a guide to its examination and to the diagnosis and treatment of its diseases*, p. 88.

McCarrison (1920). *Brit. Med. J.* i, 249.

McCollum (1917). *The Supplementary Dietary Relationship among our Natural Foodstuffs.* Paper read before the Harvey Soc. of N.Y., Jan. 13th.

Mellanby (1919). *Lancet*, i, 407.

Still (1918). *Lancet*, ii, 163.

Tibbles (1912). *Foods, their Origin, Composition and Manufacture*, p. 239.

Tozer (1921). *Biochem. J.* 15, 28.

IV. THE EFFECT OF A DIET DEFICIENT IN ANIMAL FAT ON THE BONE TISSUE (RIB JUNCTIONS) OF KITTENS.

By FRANCES MARY TOZER.

From the Department of Experimental Pathology, Lister Institute.

(*Received November 15th, 1920.*)

The following remarks are based upon the examination of the rib junctions of the kittens used by Dr H. Mackay, in the foregoing experiments [Mackay, 1921].

Dr Mackay kindly supplied me with the material before giving particulars of the treatment of each animal in order that the diagnosis of the condition of the bone tissue might be unbiassed.

The results of the examination are as follows:

1. The rib junctions of the *control animals* Nos. 2, 3 and 9, were all diagnosed as "normal" with a reservation in the case of No. 3 that the bony trabeculae appeared to be slightly shortened and less normal in appearance than those of No. 2.

It will be observed on reference to Dr Mackay's paper that No. 3 was poorly nourished whereas No. 2 was well nourished. The difference in general condition is possibly sufficient to account for and explain the slight difference in histological condition.

2. The *experimental animals* (Nos. 1, 4, 5, 6 and 7). None of these animals was diagnosed without comment as "normal." No. 1 showed a very slight disorganisation of the bony trabeculae, a pronounced and possibly abnormal increase in length of the rows of cartilage cells and a slight degree of atrophy of the marrow.

Nos. 4, 5 and 7 showed considerable shortening of the bony trabeculae, slight shortening of the rows of cartilage cells and varying degrees of atrophy of the marrow—doubtful in 4 and 5 considerable in 7.

No. 6 was nearly normal except for the condition of the marrow which resembled No. 7.

The abnormalities at the costo-chondral junction in these kittens do not appear to resemble those characteristic of rickets, the only suggestion of any connection between the two conditions being the possibly abnormal length of the rows of cartilage cells in No. 1. They do however correspond closely with the changes seen on examination of the rib junctions of guinea-pigs fed on diets deficient in fat-soluble *A*.

That deprivation of fat-soluble *A* produces marked changes at the costo-chondral junction in the guinea-pig and that these changes are moreover indistinguishable from those caused by definite, but not severe, scurvy was suggested in a previous communication [Delf and Tozer, 1918]. This suggestion has since received confirmation and a fuller account of the experiments leading to this confirmation will be published shortly in the *Journal of Pathology and Bacteriology*.

REFERENCES.

Delf and Tozer (1918). *Biochem. J.* **12**, 4.
Mackay (1921). *Biochem. J.* **15**, 19.

V. COMPARISON OF THE GROWTH-PROMOTING PROPERTIES FOR GUINEA-PIGS OF CERTAIN DIETS, CONSISTING OF NATURAL FOODSTUFFS.

By ELEANOR MARGARET HUME.

From the Department of Experimental Pathology, Lister Institute.

(*Received November 24th, 1920.*)

Introductory.

In the course of researches upon guinea-pig scurvy, which have extended over several years, evidence has gradually accumulated which seems to show that the guinea-pig, for healthy growth and maintenance, requires an abundant supply of the fat-soluble *A* accessory factor of McCollum (vitamin *A* of Drummond [1920]). Two distinct lines of evidence support this conclusion.

The first line of evidence is derived from a number of experiments in which guinea-pigs grew on one group of diets and failed to grow on another group of diets. The distribution of vitamin *A* among foodstuffs has hitherto been only partially mapped out by the various workers in that field, yet, as far as the value for that factor of the various foodstuffs used is known, those which promoted growth seem to agree in containing vitamin *A* and those which did not, in lacking it. Furthermore those foodstuffs which produced partial growth seem to have done so in proportion to their known relative value for vitamin *A*. It is true that some of the diets which did not promote growth were open to criticism in some other respects than a deficiency of vitamin *A*, but it is also true that some diets on which growth took place were equally liable to have been deficient in the same particulars and yet promoted growth. The only dietary common factor, absent from the one set of diets and present in the other set, was vitamin *A*.

Most of the diets to be described were planned for the purpose of investigating the anti-scorbutic value of certain foodstuffs and in the very earliest experiments the need for vitamin *A* to be included in the basal diet was not recognised. It was, however, soon found that on many diets the animals did not thrive [see Chick and Hume, 1917], although they did not develop scurvy, and from this time onwards 60 cc. daily of milk (autoclaved at 120° for an hour, to destroy the anti-scorbutic factor present) was made a routine addition to most experimental scurvy diets; later on, as the result of added experience, the daily ration was often increased to 90 cc. for large and quickly growing guinea-pigs.

The second line of evidence is that derived from the histological examination of the rib junctions of all the experimental guinea-pigs, which was carried out as a routine by Miss F. M. Tozer. In the course of this examination she was forced to the conclusion that a bone lesion can occur on certain diets in which there can be no possible suspicion that the anti-scorbutic ration is insufficient. This bone lesion is in some stages indistinguishable from the bone lesion of scurvy and it occurs on many different diets which however have in common a deficiency in vitamin *A*. It is described by Miss Tozer [Delf and Tozer, 1918; Tozer, 1921]. The second line of evidence agrees with and supports the first in that the bone lesion is most severe where growth is least and in that failure in growth and severity of bone lesion both appear to be inversely proportional to the amount of vitamin *A* in the diet.

It is only proposed here to bring forward the facts which form the first line of evidence, *i.e.* to set out and compare the growth occurring in guinea-pigs on certain diets, differing from one another in their content of vitamin *A*. Miss Tozer's histological diagnosis of each case, wherever such was made, will at the same time be given for comparison, by her kind permission, but a full histological treatment of the whole question will be made by her separately in another communication.

Reference to the probable need of the guinea-pig for vitamin *A* has frequently been made in the papers of Dr Harriette Chick and her co-workers at the Lister Institute [Chick, Hume and Skelton, 1918; Delf and Skelton, 1918; Chick and Campbell, 1919]. Frölich [1912] refers to a fragility of the bones, attributed to a deficient diet, which is not scurvy. Jackson and Moore [1916] speak of a lesion resembling macroscopically a rachitic rosary, produced in guinea-pigs on a diet which was not quantitatively controlled but may have been deficient either in the anti-scorbutic or in vitamin *A* or in both. Hess and Unger [1918] also refer to changes in the rib junctions of guinea-pigs, on experimental diets, which they regard as more akin to rickets than to scurvy. It is thus apparent that a number of observers have noted the occurrence of more than one bone lesion in guinea-pigs, due to dietary causes, but there does not seem to be much evidence correlating any such lesion with a deficiency of vitamin *A*.

Cohen and Mendel [1918] conclude that the fat-soluble factor is not one of the primary factors concerned in scurvy of the guinea-pig, but do not state whether they consider it may have other importance.

Technique.

The technique employed was the same as that which has been used throughout the Lister Institute scurvy experiments and has been detailed many times already. Young growing guinea-pigs about 320 g. weight were fed with a mixture of oats and wheaten bran *ad libitum*, but weighed. To this was added the food to be tested in weighed or measured quantities; the residues were either fed by hand or were measured and the quantity actually consumed

was calculated. In the present set of experiments, extra anti-scorbutic in the form of orange juice was added in those cases where the experimental ration did not already contain sufficient of that factor. It is proposed to give here details of various additions to this basal diet, together with details of the growth on such variously supplemented diets and a histological report, furnished by Miss Tozer.

The comparative growth of the animals has been set out in the form of a table, in which the weight at the start of the experiment and after 25, 35 and 45 days is given, together with the percentage increase for each animal and the average percentage increase for the whole group over each period. The normal as estimated by Delf [Delf and Tozer, 1918] is taken as the standard and in these experiments all degrees of deviation from it are found, down to cases such as Nos. 628, 629, 631, 632 (Table II, Group 28) on a diet of oats, bran, water and 10 cc. of orange juice daily, which showed no growth at all even in the first fortnight, but an actual loss in weight, amounting to an average of 19·7 % for the group for 25 days.

The histological bone lesion occurring in the rib junctions has been classified into the four very convenient degrees of severity already briefly described by Miss Tozer [Delf and Tozer, 1918]. She classifies the lesion occurring in guinea-pigs fed on a diet deficient in the anti-scorbutic or in vitamin *A* into four degrees of severity; normal, incipient, definite, acute; the exact significance of these terms can be ascertained on reference to her paper where diagrams are given. In some cases the experiments were continued for considerably longer than the 45 days for which the latest weight reading is given. In other cases the animal died after as short a period as 20 days so that the histological examination was made after the animals had been for very varying periods on the different diets. Doubtless this variation must affect to some degree the severity of the bone lesion but Miss Tozer has found that histological bone changes begin very early on a deficient diet [Tozer, 1921] and it is not therefore thought that such a variation seriously affects the comparative value of the histological findings, relative to one another. In the last column of the table the number of days elapsing between the beginning of the experiment and the date of death is noted.

Beyond the loss in weight and the bone lesion described, there are no other marked symptoms. The emaciation may be extreme, not a particle of fat being discoverable on the body in severe cases. All bones are more or less brittle and the animal is extremely prone to suffer from intercurrent disease, particularly lung affections.

Experimental Results.

The experimental results can be divided roughly into two groups, those concerned with foods which promoted growth (Table I) and those concerned with foods which did not (Table II). Naturally the division is only an artificial one, for many of the foodstuffs not promoting growth might promote

growth if given in much larger quantities and many of the growth-promoting ones would fail to do so if given in much smaller quantities, but for purposes of convenience such a division is permissible. The first or growth-promoting group includes milk in several forms, green cabbage or some modification thereof and hay. The second or non-growth-promoting group includes white cabbage and a derivative thereof, swede juice, boiled onion, orange juice and germinated peas.

Green cabbage leaf, raw. (Table I, Groups 1, 2 and 3.)

Group 1. 30 *g. daily ration.* An oat and bran diet to which 30 g. daily of raw green cabbage is added is richly growth-promoting and the average percentage increase for a group of animals for 45 days was 60·4 %, actually more than the normal standard of 55·8 %, calculated for the same period.

Histological examination of the costo-chondral junctions of the ribs. Only two animals were examined, after 39 days of the experiment, and both showed a normal histology.

Group 2. 15 *g. daily ration.* With a 15 g. daily ration, good growth but not to the full normal extent is induced. The average percentage increase for a group of seven animals for 45 days being 40·1 % which is only about two-thirds of that on 30 g.

Histological examination. Only four animals were examined; three were quite normal and the fourth was nearly so, not being sufficiently abnormal to be classed as "incipient."

Group 3. 5 *g. daily ration.* A ration of 5 g. daily also produced growth, but the increase was still further below the normal, being an average of only 29·9 % for a group of three animals for 45 days, *i.e.* about half the corresponding figure for 30 g.

Histological examination. The three animals in this group were all examined; one was not quite abnormal enough to be called incipient and the other two were between incipient and definite.

Green cabbage leaf, heated for one hour at 100°. (Table I, Groups 4 and 5.)

The cabbage was prepared by being steamed for an hour at 100°.

Group 4. 30 *g. daily ration.* On a 30 g. daily ration a group of three animals showed a rate of growth equal to that on 30 g. raw cabbage. The two animals on which the experiment was continued for 45 days showed an even better average rate of increase (71·4 %) than the rate of increase for 30 g. raw cabbage (60·4 %).

Histological examination. All three animals were examined, two of them were found to be perfectly normal and the third showed a very slight departure from normality but not sufficient to be called abnormal.

Group 5. 15 *g. daily ration.* Three animals received a daily ration of 15 g. and showed a rate of growth (45·5 %) slightly superior to that (40·1 %) for 15 g. of raw cabbage.

Table I.

Group no.	Additions to basal diet of oats, bran and water	Amount of ration	No. of animal	Initial weight g.	Wt after 25 days g.	% increase	Wt after 35 days g.	% increase	Wt after 45 days g.	% increase	Histology: Normal	Histology: Incipient	Histology: Definite	Histology: Acute	Length of life from beginning of expt. to post-mortem, days
	Normal	...	...	340	450	32·3	500	47·0	535	55·8					
1	Green cabbage leaf, raw ...	30 g.	1	285	380	33·3	415	45·6	470	64·9		Not examined			...
		,,	8	280	365	29·8	415	48·2	475	69·6		,,	,,		...
		,,	351	368	470	27·7	490	33·1	520	41·3		,,	,,		...
		,,	352	338	480	42·0	540	59·7	565	60·5		,,	,,		...
		,,	885	340	440	29·3	480	41·1	510	50·0		,,	,,		...
		,,	150	325	477	46·7	541	66·4	572	76·0		,,	,,		...
		,,	152	315	440	39·6	473	50·1	...	...	+	...	...	...	39
		,,	153	330	418	26·6	458	38·7	...	...	+	...	...	...	39
				Average ...		34·2		47·8		60·4					
2	Green cabbage leaf, raw ...	15 g.	359	350	470	34·2	480	37·1	480	37·1		Not examined			...
		,,	353	345	443	28·4	472	33·9	495	43·4		,,	,,		...
		,,	886	310	340	9·7	370	19·3	400	29·0		,,	,,		...
		,,	826	340	440	29·4	460	35·2	515	51·4		,,	,,		...
		,,	823	370	445	20·2	450	21·6	495	33·7	+	...	...	...	93
		,,	70	330	435	30·4	485	46·9	510	55·5		Not examined			...
		,,	71	305	390	27·9	375	22·9	400	31·1	+nearly	...	...	...	71
		,,	699	321	388	20·8	425	32·4	...	...	+	...	...	...	38
		,,	693 A	330	348	5·4	...	...	...	...	+	...	...	...	25
				Average ...		22·9		31·1		40·1					
3	Green cabbage leaf, raw ...	5 g.	421	330	378	14·5	390	18·1	420	27·2	...	+?	...	...	94
		,,	422	320	340	6·2	362	13·1	403	25·9	...	+ to +?		...	94
		,,	425	318	355	11·6	398	25·1	435	36·7	...	+ to +		...	94
				Average ...		10·8		18·7		29·9					
4	Green cabbage steamed 100°C. 1 hr.	30 g.	333	330	465	40·9	525	59·0	580	75·7	+ to +?		...	...	84
		,,	334	305	395	29·5	455	49·1	510	67·2	+	...	...	...	84
		,,	335	315	392	23·1	435	38·0	...	...	+	...	...	...	86
				Average ...		31·2		48·7		71·4					
5	Green cabbage steamed 100°C. 1 hr.	15 g.	338	300	385	28·3	440	46·6	465	55·0	+nearly	...	...	...	102
		,,	339	330	395	19·7	438	32·7	460	39·3		Not examined			...
		,,	340	350	405	15·7	465	32·8	498	42·2		,,	,,		...
				Average ...		21·2		37·3		45·5					
6	Green cabbage steamed 100°C. 2 hrs.	15 g.	354	320	400	25·0	430	34·3	452	38·1	+	...	...	...	96
		,,	355	330	398	20·6	430	30·3	448	35·7		Not examined			

6	Green cabbage steamed 100°C. 2 hrs.	15 g.	354	320	400	25·0	430	34·3	452	38·1	+	...	...	...	96
		,,	355	330	398	20·6	430	30·3	448	35·7	Not examined				...
		,,	356	355	413	16·5	442	24·5	470	32·3	,,	,,			...
		,,	357	340	415	22·0	430	26·4	470	38·2	+	...	...	...	90
		,,	358	330	435	31·8	452	39·9	480	45·4	+	...	...	...	95
				Average	...	23·2		30·4		37·9					
7	Raw cabbage juice, filtered ...	20 cc.	CJ 1	338	380	18·3	425	25·7	454	24·3	+nearly	...	...	...	108
		,,	CJ 2	332	350	5·4	380	14·4	380	14·4	+ to +		...	...	109
				Average	...	11·8		20·0		24·3					
8	Raw cabbage juice, filtered ...	30 cc.	CJ 10	331	410	23·8	430	29·9	440	32·9	...	+ to +		...	100
				Average	...	23·8		29·9		32·9					
9	Raw cabbage juice, unfiltered	30 cc.	CJ 9	335	403	19·4	425	26·8	440	31·0	...	+ to +		...	100
				Average	...	19·4		26·8		31·0					
10	Heated cabbage juice, filtered	20 cc.	CJ 5	329	370	12·4	316	− 3·9	292	−11·2	Not examined				...
		,,	CJ 6	332	351	5·7	369	+11·1	345	+ 3·9	,,	,,			...
				Average	...	9·0		3·6		− 3·6					
11	Heated cabbage juice, filtered +2 g. casein	20 cc.	CJ 4	330	355	7·6	303	− 8·1	342	3·6	Not examined				...
				Average	...	7·6		− 8·1		3·6					
12	Heated cabbage juice, filtered	30 cc.	CJ 3	330	400	21·2	428	29·6	456	38·3	Not examined				...
				Average	...	21·2		29·6		38·3					
13	Heated cabbage juice, unfiltered	30 cc.	CJ 7	335	380	13·4	395	16·7	312	− 6·8	Not examined				...
		,,	CJ 15	345	398	15·3	435	26·0	420	21·7	...	+ to +		...	56
				Average	...	14·3		21·3		14·9					
14	Heated cabbage juice, unfiltered +2 g. casein	30 cc.	CJ 8	331	415	25·3	435	31·4	431	30·2	...	...	+	...	64
				Average	...	25·3		31·4		30·2					
15	Heated cabbage juice, unfiltered	40 cc.	CJ 11	332	400	20·5	404	21·6	393	18·3	...	...	+	...	59
		,,	CJ 12	333	409	22·8	422	26·7	450	35·1	+ to +		...	...	121
		,,	CJ 13	332	387	13·5	389	17·4	400	20·4	+ to +		...	...	120
		,,	CJ 14	339	413	21·8	440	29·7	460	35·6	...	+	...	...	115
		,,	CJ 18	340	314	...	355	...	375	ill and recovered	+ to +		...	...	62
		,,	CJ 19	335	403	20·1	435	29·8	422	25·9	...	+ to +		...	94
		,,	CJ 21	345	370	7·2	395	14·4	...	...	...	...	...	...	...
				Average	...	17·6		23·2		27·0					
		50 cc.	CJ 21	...	...	...	...	...	398	15·3	+nearly	...	...	...	49
								Average	...	15·3					

Table I *continued*

Group no.	Additions to basal diet of oats, bran and water	Amount of ration	No. of animal	Initial weight g.	Wt after 25 days g.	% increase	Wt after 35 days g.	% increase	Wt after 45 days g.	% increase	Histology				Length of life from beginning of expt. to post-mortem, days
											Normal	Incipient	Definite	Acute	
	Normal	...	...	340	450	32·3	500	47·0	535	55·8					
16	Dried cabbage juice, green +5 cc. orange juice	50 cc.	DC 1	301	360	19·9	390	29·5	395	31·2	...	+	...	...	85
		,,	DC 2	313	370	18·2	333	6·3	347	11·1	...	+	...	...	91
		,,	DC 3	304	350	15·1	360	18·4	365	20·0	+ to	+	...	...	82
				Average ...		17·7		18·0		20·7					
17	Fresh milk + 10 cc. orange juice	20 cc.	684	334	305	− 8·6	...	...	...	...	...	...	...	...	...
		,,	685	335	350	+ 4·4	...	...	...	...	...	...	...	...	...
				Average ...		− 2·1									
18	Raw milk + 10 cc. orange juice	60 cc.	699 M	330	450	36·3	485	46·9	480	45·4	+	...	...	...	45
		,,	699 N	333	410	23·1	429	28·8	456	36·9	+nearly	+?	...	...	45
		,,	699 O	338	440	30·1	489	44·7	532	57·4	+nearly	+?	...	...	45
		,,	699 P	331	424	28·9	470	41·9	490	48·0	+nearly	...	...	...	45
				Average ...		29·6		40·5		46·9					
19	Aut. milk + 10 cc. orange juice	60 cc.	699 Q	331	455	37·4	516	55·9	532	60·7	+nearly	...	...	...	45
		,,	699 R	330	440	33·3	470	42·4	500	51·5	+nearly	...	...	...	45
		,,	699 S	340	427	25·5	445	30·8	445	30·8	...	+	...	...	45
		,,	699 T	340	428	25·8	463	36·1	464	36·4	+nearly	...	...	...	45
				Average ...		30·5		41·3		44·8					
20	Glaxo = 60 cc. raw milk + 10 cc. orange juice	60 cc.	694	337	441	30·8	466	38·2	474	40·6	...	+ to +		...	45
		,,	695	334	423	26·6	453	35·9	480	43·7	...	+	...	...	45
		,,	696	330	407	23·1	435	31·8	435	31·8	...	+ to +		...	45
		,,	697	334	403	20·6	417	24·9	450	34·7	...	+	...	...	45
				Average ...		25·2		32·7		37·7					

Histological examination. Only one animal was examined and was found nearly normal.

Steaming cabbage for an hour at 100° does not therefore appear to destroy a perceptible amount of its growth-promoting powers for guinea-pigs.

Green cabbage leaf, heated for two hours at 100°. (Table I, Group 6.)

The cabbage was prepared by being steamed for two hours at 100°.

Group 6. 15 *g. daily ration.* Five animals received 15 g. daily each and their average percentage increase (37·9 %) for 45 days, is only slightly inferior to the corresponding figure for raw cabbage (40·1 %).

Histological examination. Three of the five animals were examined and showed a normal histology.

Cabbage steamed for two hours at 100° does not appear to be materially damaged as regards its growth-promoting powers for guinea-pigs.

Filtered green raw cabbage juice. (Table I, Groups 7 and 8.)

A number of experiments were planned in order to dissociate the growth-promoting fraction, as far as possible from the proteins and other substances in the actual tissues of the leaf. Juice from raw and heated cabbage, filtered and unfiltered, was used and in one or two cases caseinogen was added to compare the effect.

For the preparation of the raw juice the green portions of the leaf, free of the mid-rib, were minced in a mincing machine, the pulp was enclosed in muslin and pressed out in a hand press. In all cases the animals were allowed to drink the juice if they would; if they would not it was fed by hand. The juice was filtered through paper.

Group 7. 20 *cc. daily ration.* Two animals received 20 cc. of the raw juice filtered and made some growth, *i.e.* an average of 24·3 % in 45 days; this is a rate of increase well below the normal (55·8 %) and less even than that (29·9 %) made on 5 g. daily of the raw green leaf.

Histological examination. Both animals were examined and one was found to be almost completely normal and the other between normal and "incipient."

Group 8. 30 *cc. daily ration.* One animal received 30 cc. daily and showed 32·9 % increase in 45 days, an increment which is still nearly 50 % below the normal.

Histological examination. The condition of the rib junctions was distinctly abnormal, being between "incipient" and "definite."

Raw filtered cabbage juice is thus capable of promoting growth but possesses this property in a much less degree than the raw leaf.

Unfiltered green raw cabbage juice. (Table I, Group 9.)

The juice used was the same as in Groups 7 and 8 except that it was unfiltered.

Group 9. 30 *cc. daily ration.* Only one animal received this ration and its percentage increase for 45 days, *i.e.* 31 %, was almost identical with that

(32·9 %) of the one animal (Group 12) which received 30 cc. of the filtered juice.

Histological examination. The histological result also corresponded with that of the animal in Group 12, being between "incipient" and "definite."

So far as evidence derived from comparison between two single animals is worth anything at all, it points to there being no loss of growth-promoting power in cabbage juice on filtration through paper.

Filtered green heated cabbage juice. (Table I, Groups 10, 11 and 12.)

The juice was prepared by steaming the cabbage at 100° for 20 minutes, folding it in muslin and pressing in a hand press. It was then filtered through a paper filter.

Group 10. 20 *cc. daily ration.* 20 cc. of the juice was fed to two animals which both showed some rise in weight at the 25th day (average 9·0 %) but this had diminished to 3·6 % on the 35th day. On the 45th day one of the two animals showed a percentage loss over the whole period of 11·2 % while the other showed some loss of what it had previously gained, though there was a net gain over the whole period of 3·9 %. The gain for this animal (No. CJ 6) for 35 days was 11·1 %.

No histological examination was made.

Group 11. 20 *cc. juice* + 2 *g. caseinogen daily ration.* The caseinogen was unpurified; it was mixed with a little water and was fed by hand. The quantity 2 g. was selected as being somewhat in excess of the amount present in 60 cc. of cow's milk, a ration of milk well known to be growth-promoting for guinea-pigs.

One animal received the ration but the caseinogen did not affect the result in its case for it just showed maintenance over the whole period of 45 days.

No histological examination was made.

Group 12. 30 *cc. daily ration.* Only one animal received 30 cc. of the filtered heated juice; for the period of 45 days it showed considerable growth (38·3 %), not equal to the normal (55·8 %), but nearly approaching that for 15 g. raw green cabbage leaf (40·1 %), and rather greater than that for 30 cc. of the raw juice either filtered or unfiltered.

No histological examination was made.

On the 20 cc. dose the juice of heated cabbage appears to be much inferior in growth promoting power to the raw juice, but on the 30 cc. dose this difference is not apparent; individual idiosyncrasy is however so great that very little importance can be attached to the behaviour of single animals, such as are available for comparison of the 30 cc. doses.

Unfiltered green heated cabbage juice. (Table I, Groups 13, 14 and 15.)

The juice was prepared as for Groups 10, 11 and 12 but it was not filtered.

Group 13. 30 *cc. daily ration.* Two animals received a 30 cc. daily ration

and both of them made about half the normal growth for 35 days, but by the 45th day one animal, CJ 15, had begun to lose slightly and the other, CJ 7, had converted the previous gain into a negative balance of 6·8 %. In such cases as this the dose would appear to be sufficient to start growth but ceases to be so when the animal is larger. It can scarcely be that the animal starts growth on a reserve of its own which gradually becomes exhausted, as seems to be the case with the rat, when suffering from a deficiency of vitamin *A*, because on some of the diets, such as oats and bran and orange juice (Table II, Group 28), there is absolutely no increase in weight at all but a rapid decline from the start.

Histological examination. The one animal, CJ 15, which made the better growth gave a histological result between "incipient" and "definite"; the other animal was not examined.

Group 14. 30 *cc. juice* + 2 *g. caseinogen, daily ration.* This group only contained one animal which made rather better growth than the two animals in the preceding group; at 35 days it showed an increase of 31·4 % but by the 45th day it also had begun to decline and showed a net increase for the whole period of only 30·2 %.

Histological examination. The lesion in the rib junctions was of the third degree, being "definite."

It is unlikely that the rather better growth shown in this case on the addition of caseinogen was due to more than the individual idiosyncrasy of the animal; the addition of caseinogen in Group 2 produced no such effect.

Group 15. 40 *cc. daily ration.* This group includes seven animals, of which one was ill during the experiment but recovered, the values for it are therefore given but are not included in the averages. The group for each of the three time periods shows a rate of increase about half the normal.

One animal (CJ 21) which showed a low rate of growth had the daily dose increased to 50 cc. after the 35th day but this increase was not reflected in the subsequent rate of growth although the histology was almost perfectly normal.

Histological examination. The histological results were better than those recorded for any of the other cabbage juice groups, excepting Group 7 (20 cc. raw green filtered juice). One animal showed an almost normal histology, three were between normal and "incipient," one was "incipient," one was between "incipient" and "definite" and one was "definite."

Taken together the cabbage juice results indicate that filtration through paper does not affect the growth-promoting properties of cabbage juice but that the juice from the raw leaves is more potent than that from the cooked leaves. As it was observed, in experiments already detailed, that steaming did not appreciably diminish the value of the leaves when fed whole, it must be that the growth-promoting principle does not pass out with the juice on pressure as readily after heating as it did before. Perfectly normal growth was not obtained in any case with the cabbage juice rations but the indica-

tions are that such growth would be secured if larger doses of the juice could be conveniently given to the animals.

Dried cabbage juice. (Table I, Group 16.)

The juice was prepared from steamed cabbage leaves in the manner already described but was not filtered. The yield of juice was about 50 % of the weight of cabbage taken. It was poured into large flat dishes which were placed in a hot room (37°) with an electric fan just beside them. After about 24 hours the liquid had evaporated to a sticky, thick paste and was further dried by the method described by Harden and Robison [1919]. The paste was scraped up and worked up with a knife so as to become full of air bubbles. The aerated mass was placed in a desiccator which was then evacuated. The whole mass swelled and rose up and if left in the desiccator was dry and porous by the next morning and could be broken up into a coarse powder. Frequently however the drying was not complete in one operation and it was necessary to work the mass up and evacuate the desiccator several times. The dry solids generally amounted to about 5 % of the weight of juice taken. The powder was extremely hygroscopic and had to be kept in a desiccator; new batches were made about once a fortnight. For administration to the guinea-pigs, the powder was mixed with a little water and given by hand.

With this ration 5 cc. of orange juice was given daily, as the anti-scorbutic value of the cabbage juice was likely to have suffered seriously in the process of drying.

Group 16. *50 cc. daily ration.* The dried cabbage juice was fed to three guinea-pigs in amounts calculated to be equivalent to 50 cc. of the heated juice. Growth was obtained but was less than one-half the normal and was also less than that obtained on smaller doses of the heated juice which had not been dried.

Histological examination. The results were also rather inferior to those on smaller doses of the heated juice. One animal was between normal and "incipient" and two were "incipient."

There is some loss of growth-promoting power on drying cabbage juice but the loss is by no means complete.

Raw cow's milk. (Table I, Groups 17 and 18.)

The milk used in these experiments was very pure, freshly delivered certified country milk; the time of year at which the experiments were performed was unfortunately not the same in every case but the months are given in each case. 10 cc. orange juice daily was given with this and all other milk rations, to supply the anti-scorbutic factor which would otherwise have been insufficient.

Group 17. *20 cc. daily ration, increasing to 40 cc. and later to 60 cc. Dec.–Feb.* Two animals received 20 cc. daily and after 25 days one, No. 684, showed a loss of 8·6 % and the other, No. 685, showed a gain of 4·4 %.

After 30 days the amount of milk was increased to 40 cc. daily and after 30 days on this altered ration No. 684 had recovered a very little weight but had not regained its original weight. No. 685 had gained steadily, though not normally, and was allowed to remain on the 40 cc. ration on which it continued to gain slowly for over 80 days. No. 684, however, was altered to 60 cc. daily on which after a little delay it made a sharp gain in weight.

No histological examination was made.

From experiments on the anti-scorbutic values of foodstuffs published elsewhere [Chick and Hume, 1917] it is well known that 60 cc. of autoclaved milk daily gives very good, if not perfectly normal growth in guinea-pigs, when added to a diet of oats and wheaten bran and orange juice. The foregoing experiment was sufficient to orientate and to show that a quantity less than 60 cc. of raw milk will not do so. This amount was therefore chosen as the daily ration in the following experiments, Groups 18, 19, 20 and 21, in which the growth-promoting powers of several kinds of milk were examined.

Group 18. 60 *cc. daily ration. June–July.* Four animals received 60 cc. daily of the raw milk and made good but not quite normal growth, the average increase for the group for 45 days being 46·9 % as against 55·8 % for the normal.

Histological examination. Of the four animals one was perfectly normal and the remaining three were almost completely so.

It would therefore appear that 60 cc. raw milk a day is almost but not quite a sufficient quantity to promote normal growth.

Cow's milk autoclaved for one hour at 120°. (Table I, Group 19.)

The same milk as that used in Groups 17 and 18 was autoclaved for one hour at 120°.

Group 19. 60 *cc. daily ration. June–July.* Four animals receiving the above ration showed a rate of growth (44·8 %), almost identical with that (46·9 %) of the animals which received 60 cc. raw milk.

Histological examination. The results were very slightly inferior to those of animals in Group 18, three being nearly normal and one "incipient."

It therefore appears that heating for an hour at 120° in an autoclave does not appreciably damage the growth-promoting power of milk for guinea-pigs.

Dried milk. (Table I, Group 20.)

A well-known brand of dried milk, much used in infant feeding, was employed. It was not more than a month old and was prepared in March and April. The milk was made up so that the liquid should contain 3·5 % of fat but the powder is a proprietary article in which the original composition has been slightly modified. No fine quantitative deductions could therefore be drawn from the result, but it should be possible to say whether drying seriously damages the growth-promoting properties of milk for guinea-pigs or no.

Group 20. *60 cc. daily ration.* Four animals received the 60 cc. daily ration and showed over a period of 45 days a rate of growth (37·7 %), slightly inferior to the corresponding rate (46·9 %) for raw milk.

Histological examination. The results were distinctly inferior to those on raw milk, two animals being "incipient" and two between "incipient" and "definite."

Since the dried milk was not prepared from the milk used in the raw milk experiments and since the time of year and consequently the food of the cows were different, it is not possible to attribute the slight inferiority of the dried milk, with certainty, to the fact that it had been dried. It is possible however to say that if drying affects the growth-promoting power of milk for guinea-pigs at all, it only does so very slightly.

Hay autoclaved at 120° *for one hour.*

No continuous experiments were put up with a daily ration of hay, but six animals receiving oats and bran and varying rations of lemon juice, and ten animals receiving oats and bran and varying rations of germinated peas showed no appreciable growth. Autoclaved hay (about 20 g. daily) was then added to the diets for four days and every animal showed an immediate sharp rise in weight which at once ceased when the hay was taken away again.

After drying and autoclaving for an hour at 120°, therefore, hay still retains considerable growth-promoting power for guinea-pigs, though the above observation gives no indication whatever, whether some of the original growth-promoting power is lost or not.

White cabbage leaf, raw. (Table II, Group 21.)

The first of the non-growth-promoting group of foodstuffs is the raw white heart of cabbage. Very tightly closed cabbage hearts were used and the outer green leaves were stripped off until inside leaves of a uniform pale etiolated greenish yellow were reached.

Group 21. *15 g. daily ration.* Guinea-pigs do not willingly eat the heart leaves of cabbage but two were persuaded to do so. One, No. 698, failed altogether to grow and when the experiment ended after 39 days showed a loss of 4·3 %. The other, No. 692, showed a gain of 4·4 % at the 35th day, which was converted into a loss of 2·4 % by the 45th day.

Histological examination. The lesion was well marked being between "incipient" and "definite" for one animal and "definite" for the other.

Since even as little as 5 g. of green raw cabbage have been shown to produce as much as half the normal growth, while 15 g. of white cabbage produce practically no growth at all, it is clear that white cabbage is vastly inferior to green in its growth-promoting properties. When however the state of affairs on a white cabbage ration is compared with that on oats, wheaten bran and orange juice it is apparent that the white cabbage cannot be so completely deficient in growth-promoting properties as is the orange juice. The

Table II

Group	Addition to basal diet of	Amount of	No. of	Initial weight	Wt after 25 days	Wt after 35 days	Wt after 45 days	Histology	Length of life from beginning of expt. to post-mortem

Group no.	Addition to basal diet of oats, bran and water	Amount of ration	No. of animal	Initial wt. g.	Wt after 25 days g.	% increase	Wt after 35 days g.	% increase	Wt after 45 days g.	% increase	Histology				Days from beginning of expt. to post-mortem, days
											Normal	Incipient	Definite	Acute	
	Normal	...	...	340	450	32·3	500	47·0	535	55·8					
21	White cabbage leaf, raw ...	15 g.	692	337	333	− 1·1	352	+ 4·4	330	− 2·4	...	...	+	...	48
		,,	698	320	299	− 6·5	306	− 4·3	...	...	...	+ to	+	...	40
				Average ...		− 3·3		0·3		− 2·4					
22	Heated, white cabbage juice, unfiltered	40 cc.	CJ 17	340	380	10·1	345	+ 1·4	(250) dead	...	...	+ to	+	...	41
		,,	CJ 20	328	315	− 4·0	287	−12·5	(250) dead	...	...	...	+	...	47
		,,	CJ 16	342	373	9·0	392	14·6	328	− 4·0	...	...	+*a*	+*b*	62
				Average ...		5·0		1·1		− 4·0					
23	Onion, steamed	30 g.	62	300	350 23 days	+16·6	dead	...	...	...		Not examined			...
		,,	63	300	355	+18·3	370	23·3	360	20·0	...	...	+	...	57
		,,	64	330	295	−10·6	305	− 7·5	300	−10·0	...	...	+	...	49
		,,	65 A	370	350	− 5·4	315	−14·8	230	−37·8	...	+?	+?	...	45
				Average ...		+ 4·7		− 0·3		− 9·2					
24	Onion steamed	60 g.	120 A	280	270	− 3·6	275	− 1·7	270	− 3·6	...	...	+	...	58
				Average ...		− 3·6		− 1·7		− 3·6					
25	Swede juice	20 cc.	536	323	312	− 3·4	318	− 1·5	275	−17·0	...	...	+?	...	53
		,,	537	340	302	−11·1	286	−15·8	246	−27·6	...	...	+	...	43
		,,	538	340	320	− 5·8	299	−12·0	240	dying (−29·4) dead	...	...	+	...	42
		,,	539	325	358	10·1	350	7·6	311	− 4·3	...	+	...	...	54
				Average ...		− 2·5		− 5·4		−19·8					
26	Germinated peas	20 g.	308	420	430	+ 2·3	420	0	405	− 3·5		Not examined			...
		,,	309	345	330	− 4·3	...	...	...	...		,,	,,		...
		,,	310	390	380	− 2·5	...	...	...	...		,,	,,		...
				Average ...		− 1·5				− 3·5					
27	Germinated peas	30 g.	312	380	360 20 days	− 5·2	...	...	...	...		Not examined			...
		,,	315	325	320 23 days	− 1·5	...	...	...	...		,,	,,		...
				Average ...		− 3·3									
28	Orange juice	10 cc.	628	340	297	−12·6	...	...	...	...	...	...	+	...	...
		,,	629	325	259	−20·3	...	...	...	...	...	...	+	...	...
		,,	631	341	278	−18·4	...	...	...	...	...	...	...	+	...
		,,	632	340	246	−27·8	...	...	...	...	...	...	+	...	...
				Average ...		−19·7									

former at least secures something like maintenance for as much as 45 days, whereas on the orange juice there is a heavy loss (average 19·7 %, Group 28, Table II), even in 25 days.

Unfiltered heated white cabbage juice. (Table II, Group 22.)

The juice was prepared in the same way as the juice of steamed green cabbage leaves and the leaves were selected as in the foregoing experiment (Group 21). The juice was not filtered.

Group 22. 40 *cc. daily ration.* Three animals received the above ration; of these one showed no growth but a negative balance of 4 % even by the 25th day; on the 35th day the negative balance was increased to 12·5 % and before the 45th day the animal was dead. The other two animals, CJ 16 and CJ 17, showed some increase (9 and 10 %) on the 25th day, but by the 35th day CJ 17 was losing weight and was dead before the 45th day; CJ 16 went on gaining a little to the 25th day (14·6 %) but by the 45th day showed a negative balance of 4 % and died shortly afterwards.

Histological examination. One animal was between "incipient" and "definite," one "definite" and one between "definite" and "acute."

Comparison of the 40 cc. dose of the green (Group 15) and of the white cabbage juice shows the great inferiority of the latter. On the green, growth amounting to about half the normal was obtained; on the white, after slight initial growth the animals declined and died in 45 days or less.

Steamed Onion. (Table II, Groups 23 and 24.)

The onion was steamed in a double saucepan for a rather indeterminate time, probably about one hour; it is however known from the experiments with steamed cabbage that steaming even for two hours is unlikely greatly to damage the growth-promoting property if present.

Group 23. 30 *g. daily ration.* Four animals received this ration and did not give a very uniform result. Two, Nos. 62 and 63, showed a considerable rise, nearly half the normal in the first 25 days but one of these declined and was dead very soon after. No. 63 increased a little further to the 35th day (23·3 %) but then began to decline very slowly, showing a net increase of only 20 % on the 45th day. The other two, Nos. 64 and 65 A, both showed a constantly increasing loss amounting to 10 % in one case and 37·8 % in the other, by the 45th day.

Histological examination. Three of the five animals were examined; one was between "incipient" and "definite" and two were "definite."

Group 24. 60 *g. daily ration.* One animal received a daily ration of 60 g. on which it showed no gain but almost maintained itself for 45 days.

Histological examination. The lesion was "definite."

Steamed onion does not seem to be wholly devoid of growth-promoting power for guinea-pigs but in most cases even in large rations it produces very little effect.

Swede juice. (Table II, Group 25.)

The juice of the swede turnip (*Brassica campestris*, var. *Napo-Brassica*) was expressed by first grating the swede root on a kitchen grater and then enfolding the pulp in muslin and squeezing out the juice by hand.

Group 25. 20 *cc. daily ration.* Four animals received the ration and three showed a decline from the start; of these two were dead by the 45th day. The fourth, No. 539, showed an increase of 10·1 % on the 25th day, but by the 35th day the net increase was only 7·6 % and by the 45th day there was a negative balance of 4·3 %.

Histological examination. One animal was "incipient" and three "definite."

Swede juice is evidently almost devoid of growth-promoting power for guinea-pigs in quantities of 20 cc. daily.

Germinated peas. (Table II, Groups 26 and 27.)

Peas were germinated by soaking in water for 24 hours and germinating them in a funnel for 48 hours; the outer coat of the seed was removed and the peas fed to the guinea-pigs whole.

Group 26. 20 *g. daily ration.* Three animals received the ration for 25 days and just maintained weight; only one received it for the full 45 days and showed a very slight negative balance.

No histological examination was made.

Group 27. 30 *g. daily ration.* Two animals were used but the experiment only lasted 20 days in one case and 23 in the other, both animals showed slight negative balance.

No histological examination was made.

Germinated peas appear to possess very little growth-promoting power for guinea-pigs.

Orange juice. (Table II, Group 28.)

The oranges were cut across and the juice expressed by hand.

Group 28. 10 *cc. ration daily.* Four animals received the ration and after 25 days showed a large negative balance, amounting to an average for the group of 19·7 %.

Histological examination. The lesion was very bad, three animals being "definite" and one "acute."

The dose of orange juice given was only a small one, not sufficient to amount to a real test of the growth-promoting properties of orange juice; the result with these four animals is only quoted here to show that there is no gain at all but an immediate and rapid loss on the most deficient type of diet.

Discussion and Conclusions.

It is believed that the foregoing experiments indicate that the guinea-pig requires for growth and maintenance a considerable quantity of the fat-soluble accessory factor of McCollum (vitamin *A* of Drummond) in its diet.

Such a conclusion is reached for two reasons, firstly the diets which produced growth were not superior in their protein, salt or caloric value, to those which did not; the difference must therefore lie in their vitamin content, but it is known that the anti-scorbutic and anti-neuritic vitamins were adequately supplied in all cases; the only known dietary constituent which could have been lacking is thus the vitamin *A*.

Secondly, the distribution of the growth-promoting factor revealed in these experiments, and its behaviour under certain forms of treatment, correspond with what is known of the distribution of vitamin *A* and its behaviour in similar circumstances.

To take the first point in more detail, growth occurs on green cabbage juice but not on white nor on swede juice, it occurs on green cabbage leaf (5 g.) but not white (15 g.) nor on germinated peas nor on onions. These sets of rations do not differ materially from one another in salt, protein or caloric value, the difference must rather be that of a body, present only in the minute amounts in which a vitamin is present. If it be granted then that the growth-promoting factor in this set of experiments is probably a vitamin, it is further apparent from the next paragraph that that vitamin corresponds with vitamin *A* in its distribution among foodstuffs and in its behaviour.

The work of McCollum has shown that vitamin *A* is present in green leaves [McCollum, Simmonds and Pitz, 1916] and in the butter fat of milk [McCollum, Simmonds and Pitz, 1916; McCollum and Davis, 1919]; in his book, McCollum [1920, p. 89] makes the generalisation, drawn from various experiments, that it is only present to a small extent in most roots and non-fatty seeds, such as cereals. Osborne and Mendel [1919] show that it is still present in green leaves after drying. Correspondingly it is found in the experiments detailed in this paper, that green cabbage and milk and dried leaves (hay) promote growth in guinea-pigs, while roots, as represented by swede juice and onions (truly an underground stem), and seeds, as represented by peas, fail to do so.

Coutts and Winfield [1918] have shown that milk, after drying, is still a rich source of vitamin *A* for rats and similarly in these experiments, dried milk is found to be little if at all inferior to raw milk in its growth-promoting properties.

Till recently the evidence with regard to the behaviour of vitamin *A* towards heat was conflicting but the position is now greatly cleared up by a contribution of Hopkins [1920], which shows that vitamin *A* is resistant to heat only as long as there is no access of oxygen but that as soon as oxidation is permitted at the time of heating, destruction of vitamin *A* begins to take place. In the present experiments in which milk and cabbage were heated in an autoclave or in a steamer, little or no loss of growth-promoting properties could be detected and correspondingly it is clear that the conditions were not such as would promote oxidation.

Lastly Drummond and Coward have found (Unpublished Experiments) that etiolated and non-green organs of plants are deficient in vitamin *A*,

corresponding with the present results that they are non-growth-promoting for guinea-pigs.

The correspondence is complete as far as the experiments go and there are no discordant facts, but on the other hand the evidence is not quite complete, for in no case can it be said that the *only* difference between a growth-promoting and a non-growth-promoting diet was the presence or absence of vitamin *A*; were it shown for instance that milk on heating with access of oxygen loses at the same time its growth-promoting power for guinea-pigs and its vitamin *A* value for rats, the evidence could be regarded as conclusive.

Prima facie it is extremely probable that the guinea-pig would require vitamin *A* for it is akin to the rat and must be accustomed, in natural conditions, to a diet of greenstuff even richer in vitamin *A* than the diet of the wild rat; such a line of argument is not however really permissible, for the guinea-pig differs, on the other hand, very markedly from the rat in its large need of the anti-scorbutic factor, a need so large that it has probably limited the creature's geographical distribution and made the numerical success achieved by the rat, mouse and rabbit in the struggle for existence, an impossibility for it.

If it can be accepted from these experiments that the guinea-pig is an animal requiring a large supply of vitamin *A*, it provides another animal on which the vitamin *A* value of certain classes of foodstuffs can be tested. The technique of rat feeding for testing the value of substances in vitamin *A* has now been much improved by the work of Drummond and Zilva but it still holds many difficulties and when the vitamin *A* value of a substance is low, so that large quantities of it have to be given, the technique sometimes fails altogether and frequently from the quantitative point of view. It is exceedingly difficult to hand-feed an unwilling rat, it is comparatively easy to do so with a guinea-pig. Further, guinea-pigs do not appear to grow at all if the diet is entirely deficient in vitamin *A*, though perhaps a larger number of experiments is needed, before this can be affirmed absolutely to be the case. If, as is believed, it is the case, then the growth achieved on any diet can be regarded as a measure of the amount of vitamin *A* in that diet and even the first few days of growth or failure can be regarded as the true trend of the whole of the rest of the experiment, without the need of any preliminary depletion period such as it is often customary to use with rats. In this way, vegetables, fruits, extracts of substances and other non-fatty preparations can be tested on guinea-pigs for their vitamin *A* value, but, as far as can be seen, fats cannot be so tested. Fat as emulsion in milk is tolerated by the guinea-pig but for more concentrated fats it appears to have an intolerance; cream and egg yolk were found to be fatal and codliver oil, even in small doses, appeared to have a very deleterious effect; these substances are however ones with which the rat technique is able to deal.

The above experiments were all conducted under the direction of Dr

Harriette Chick by whom I am kindly permitted to collect and tabulate them. Almost every one of those who have ever cooperated with Dr Chick, in her work on deficiency diseases, has contributed to the actual performing of these experiments and I have to thank Dr Dalyell, Miss Rhodes, Miss Skelton and particularly Mrs Barnes and Dr Delf for permission to use them. A number of them have been published already in various papers on guinea-pig scurvy, particularly those of Dr Delf on cabbage [Delf and Skelton, 1918; Delf and Tozer, 1918], the question of the effect of heat on the growth-promoting properties of cabbage is there discussed but it is not thought out of place to quote the same experiments again here.

Summary.

(1) A large number of dietary experiments on guinea-pigs, originally planned to determine the anti-scorbutic values of various foodstuffs, are analysed and the diets tabulated together with the growth of the animals on the diets.

(2) Growth is found to take place when green cabbage (raw or steamed), green cabbage juice, hay and milk (raw, heated or dried) are added to a diet of oats and bran and water. Orange juice was added when the anti-scorbutic value of the ration was insufficient. Growth is found to vary according to the size of the ration given.

(3) Little or no growth is found to take place when raw white cabbage, white cabbage juice, swede juice, orange juice, onion or germinated peas are added to the diet.

(4) It is argued that the diets could only have been deficient in a dietary factor of the rank of a vitamin and that the growth-promoting factor in these experiments corresponds in its distribution among foodstuffs with the known distribution of vitamin *A*.

(5) As the result of these experiments guinea-pigs are recommended for trial, as suitable for testing the vitamin *A* value of non-fatty foodstuffs, for which rats are for any reason unsuitable.

REFERENCES.

Chick and Campbell (1919). *Lancet*, **97**, ii, 320.
Chick and Hume (1917). *Trans. Soc. Trop. Med. and Hyg.* **10**, 141.
Chick, Hume and Skelton (1918). *Biochem. J.* **12**, 135.
Cohen and Mendel (1918). *J. Biol. Chem.* **35**, 425.
Coutts and Winfield (1918). *Report to the Local Government Board.* New Series 116. Food Reports 24.
Delf and Skelton (1918). *Biochem. J.* **12**, 448.
Delf and Tozer (1918). *Biochem. J.* **12**, 416.
Drummond (1920). *Biochem. J.* **14**, 660.
Frölich (1912). *Zeitsch. Hyg.* **72**, 165.
Harden and Robison (1919). *Roy. Army Med. Corps J.* **32**, 50.
Hess and Unger (1918). *J. Biol. Chem.* **35**, 480.
Hopkins (1920). *Brit. Med. J.* ii, 147. *Biochem. J.* **14**, 725.
Jackson and Moore (1916). *J. Infec. Dis.* **19**, 478.
McCollum (1920). *The Newer Knowledge of Nutrition.* Macmillan, New York, 1920.
McCollum and Davis (1919). *J. Biol. Chem.* **37**, 187.
McCollum, Simmonds and Pitz (1916). *Proc. Soc. Exp. Biol. Med.* **13**, 129.
Osborne and Mendel (1919). *J. Biol. Chem.* **37**, 187.
Tozer (1921). *Biochem. J.* **15**, 28.

VI. A HUMAN ENTEROLITH CONTAINING CHOLEIC ACID.

By HENRY STANLEY RAPER.

From the Department of Physiology and Biochemistry, the University of Leeds.

(*Received November 23rd, 1920.*)

The enteroliths occurring in man which have been described previously have consisted largely of inorganic material, principally ammonio-magnesium phosphate, calcium phosphate and calcium carbonate. Concretions in the alimentary tract may also occur, however, which contain notable quantities of calcium or magnesium soaps [Williams, 1907], and yet others which contain gall stones as a nucleus.

The enterolith described in the present paper was found by Mr James Phillips, Honorary Surgeon to the Bradford Royal Infirmary, in a case operated on for intestinal obstruction and he kindly allowed me to examine it. The stone was found about the middle of the jejunum and was the cause of the obstruction. In the ordinary way it might have been mistaken for a gall stone but was more friable than gall stones usually are. It weighed 6·8 g., was about the size of a walnut and on section had no definite nucleus. When ground up it had a yellowish sand colour. From the ground material two small pieces of tissue resembling fruit skin were separated. On heating a small portion on platinum foil the material was seen to be almost entirely organic, only a trace of ash being left. An actual estimation of ash showed that only 1·19 % was present.

A preliminary extraction with solvents showed that a portion was easily soluble in ether and light petroleum; another portion was slightly soluble in ether, more so in acetone and very soluble in alcohol. After removing all the soluble matter with ether and alcohol a pigmented residue was left which gave bile pigment reactions and contained food debris (starch and protein colour reactions). A quantitative examination by extraction of a weighed amount of the material in a Soxhlet apparatus was next carried out in order to determine the amount of the various fractions. The amount of material used for the extractions was 1·8896 g.

Ether Extract. This extraction was not carried to completion but allowed to proceed until it was judged that all the easily soluble substance had been removed. After distilling off the ether the residue was extracted several times

with hot light petroleum to remove fatty acids, fat and cholesterol-like substances. The residue from the petroleum extract weighed 0·3094 g. and represented 16·37 % of the original material. It was dissolved in alcohol, the fatty acids neutralised with sodium hydroxide and the alcoholic solution diluted with water until it contained about 50 % alcohol. The fat and unsaponifiable matter were then extracted by light petroleum and weighed. 0·0561 g. was obtained, which is equal to 5·52 % of the original material. From the alcoholic solution of the soaps the fatty acids were recovered and their iodine value determined. 0·2233 g. absorbed 0·1022 g. I; iodine value, 45·8. That part of the ether extract which was insoluble in light petroleum was easily soluble in alcohol and was therefore added to the next extract, which was obtained by continuing the extraction in the Soxhlet apparatus with alcohol. The total alcohol-soluble substance thus obtained weighed 1·37 g. and represented 72·5 % of the original material. Its further examination is described later. The residue from the enterolith which was insoluble in alcohol or ether weighed 0·16 g., and formed therefore 8·4 % of the whole. It gave the usual colour reactions for proteins and on extracting with boiling water a solution was obtained which gave a good reaction for starch. On extracting some of the material with acid alcohol and applying Cole's modification of Huppert's test a strongly positive reaction for bile pigments was obtained. This residue therefore consisted of food debris mixed with bile pigment.

Alcohol Extract. As indicated above, this formed the main portion of the enterolith. It was insoluble in water, easily soluble in hot alcohol and moderately soluble in hot acetone. It crystallised easily from hot aqueous alcohol (75 %), and after recrystallisation melted at 185°–186° (corr.). It was decolorised by boiling with charcoal and by further recrystallisation was obtained in colourless rosettes of prisms melting at 186·5°–187·5°. This melting point did not change on further recrystallisation. The substance did not contain nitrogen, it was an acid, since it dissolved easily in aqueous alkali and it gave Pettenkofer's reaction for bile salts. It was therefore suspected to be an unconjugated bile acid. Further investigation confirmed this view and it was identified as choleic acid. The melting point of choleic acid is usually given as 185°–187° [Schryver, 1912; Fischer and Meyer, 1911; Wieland and Sorge, 1916], whereas that found in the present instance was slightly higher and more sharp. To confirm the identity of the acid a mixed melting point was determined. Dr Schryver kindly furnished me with a specimen of choleic acid prepared by him from ox bile and a portion of this, after recrystallisation until its M.P. was constant, was mixed with an equal amount of the acid from the enterolith. The two were fused, the mass ground up and the M.P. of the mixture determined. It was 186°–187°. The two acids were therefore identical.

The optical activity was determined in a 2·316 % solution in absolute alcohol. Length of tube 1 dcm., temp. 20°, rotation observed + 1·17° hence

$[\alpha]_D^{20} = +50{\cdot}52°$. The values previously recorded for choleic acid are slightly lower than this, the highest being + 48·72°.

On combustion the following results were obtained:

	Found	Calc. for $C_{24}H_{40}O_4$	Calc. for $C_{24}H_{42}O_4$
C	72·92, 73·28	73·47	73·10
H	10·53, 10·78	10·20	10·66

Fischer and Meyer [1911] give $C_{24}H_{40}O_4$ as the formula for choleic acid but the analytical figures obtained in this case agree better with the formula $C_{24}H_{42}O_4$ as also do those of Fischer and Meyer.

The molecular weight was determined by titration with $N/10$ NaOH, the acid being dissolved in neutral alcohol, and was found to be 384·9. Calculated for $C_{24}H_{42}O_4$, 394.

According to Wieland choleic acid is a compound of eight molecules of desoxycholic acid and one molecule of a higher fatty acid. The figures obtained in the combustion analysis are not incompatible with this view except that the carbon figure is somewhat low, neither is the value obtained for the specific rotation.

The acid was tasteless like that isolated from ox gall stones by Fischer and Meyer. It was also observed that the choleic acid sent by Dr Schryver, when quite pure was also tasteless. Some observers have described choleic acid as having a bitter taste but small traces of cholic acid might have been responsible for this.

There appears to be no doubt that the acid from the enterolith was choleic acid. Being unassociated with other bile acids in any considerable quantity it was easily purified and probably on this account had a slightly higher melting point and specific rotation than other workers have observed.

After the choleic acid had been crystallised out, an examination of the mother liquors was made in order to determine whether desoxycholic acid was present. On evaporation of the mother liquor and crystallisation of the residue from glacial acetic acid a very small amount of an acid, M.P. 139·5°–142·5°, was obtained. Desoxycholic acid melts at 145° and according to Wieland and Sorge [1916] choleic acid when heated with glacial acetic acid forms a compound melting at 144°–146°; it was not possible therefore with the small amount of acid obtained to establish its identity.

It may be concluded therefore that the enterolith had the following composition:

Fatty acids	10·85 %
Neutral fat and unsaponifiable substances	5·52 „
Unconjugated bile acids (almost wholly choleic acid)	72·5 „
Food residues	8·4 „

It was found later that the simplest method of getting the choleic acid from the calculus was to extract the latter with acetone in a Soxhlet apparatus. The acetone extract, after removal of the acetone, was boiled with light petroleum to remove fatty acids and fat. There was then left behind a residue,

insoluble in light petroleum, consisting largely of the bile acid. This was found to be practically pure after a single crystallisation from aqueous alcohol.

I believe this is the first recorded instance of the occurrence of an intestinal calculus composed largely of one of the unconjugated bile acids. Choleic acid has been detected in ox gall stones by Fischer and Meyer, but, so far as I am aware, it has not been found in the ordinary cholesterol stones of man. There is no evidence in the present case that the enterolith originated in the gall bladder. Although cholesterol was not definitely isolated from it the amount present must have been very small since the unsaponifiable matter soluble in light petroleum was only 5 %. Further, the bile acid found was unconjugated and had probably arisen from the conjugated acid by bacterial action in the small intestine. Since cholic, choleic and desoxycholic acids are all relatively insoluble in water it is difficult to understand how preferential deposition of one of these acids rather than the other two should take place unless it be assumed that the acid found was the only one being formed by the liver. The existing analyses of human bile from various individuals do not furnish any ground for such an assumption and it certainly seems very improbable. What appears more likely in the present case is that glycocholeic acid, which is more insoluble than glycocholic acid, was deposited in the first instance and subsequently decomposed by bacteria giving rise to choleic acid.

Cases of intestinal obstruction due to gall stones which have ulcerated through the gall bladder into the small intestine are well known. It seems possible however, as pointed out by Phillips [1921], that some of the cases of intestinal obstruction supposedly due to gall stones may be due to deposits of choleic acid similar to that described in this paper. In appearance, such deposits are not unlike gall stones.

REFERENCES.

Fischer and Meyer (1911). *Zeitsch. physiol. Chem.* **66**, 95.
Phillips (1921). *Brit. J. Surg.* **8**, 378.
Schryver (1912). *J. Physiol.* **44**, 265.
Wieland and Sorge (1916). *Zeitsch. physiol. Chem.* **97**, 1
Williams (1907). *Biochem. J.* **2**, 395.

VII. THE NEPHELOMETRIC ESTIMATION OF QUININE IN BLOOD.

By HUGH WILLIAM ACTON and HAROLD KING.

From the Department of Biochemistry and Pharmacology, Medical Research Council.

(*Received December 3rd, 1920.*)

In a group of papers, Ramsden and Lipkin [1918], Ramsden, Lipkin and Whitley [1918] and Lipkin [1919] have described the chemical assay by a nephelometric method of traces of quinine when present in tissues and body fluids. The method consists essentially in matching the turbidity produced in the quinine solution of unknown strength by the addition of Tanret's reagent (potassium mercuric iodide) with the turbidity produced in a series of standard solutions. The sensitiveness of the test is enhanced by producing the turbidity in saturated ammonium sulphate solutions, preliminary trial having indicated the dilution necessary to bring the solution of unknown strength within the range of the standard solutions, usually between 1 in 200,000 and 1 in 300,000.

One of us [Acton, 1920] having found other cinchona alkaloids of great value clinically in the treatment of malaria, it was thought that some light might be thrown on the mode of action of quinine in malaria by the interpretation of the results obtained by using the afore-mentioned method for determining the relative distribution of the various cinchona alkaloids in the body.

That results of practical significance might be obtained by such an investigation seemed to be suggested not only by Ramsden and Lipkin's results but more particularly by the recent work of Morgenroth [1918], who has based an ingenious theory of the action of quinine, on the distribution of this alkaloid and of the closely related alkaloid optoquine (ethylhydrocupreine) between the corpuscles and plasma as determined by himself by physiological methods—production of anaesthesia in the rabbit's cornea and inhibition of the growth of bacteria. According to Morgenroth's determinations by these methods quinine and the related alkaloids, when added to blood, accumulate in or on the red corpuscles, the serum or plasma retaining only a low proportion.

This statement of Morgenroth is in pronounced disagreement with the various determinations of the distribution of quinine in blood which have been made by chemical methods. Hartmann and Zila [1918] found a distribution between plasma and corpuscles in the ratio of 8 to 5. Halberkann

[1919], using larger quantities of quinine, found 35 to 48 %, and in one case as much as 54 % in the corpuscles; but neither of these observers showed a predominance in the corpuscles such as that suggested by Morgenroth's results. Even more definitely in disagreement with Morgenroth's findings were those of Ramsden, Lipkin and Whitley, who found a distribution between serum and corpuscles in favour of the serum in the proportion of from 3 : 1 to 2 : 1. More recent determinations by Lipkin [Ramsden, 1920] have again shown a proportion of 3 : 1 in favour of the serum.

It seemed desirable to make an effort to clear up this discrepancy as regards quinine and to apply to a study of the distribution of the other cinchona alkaloids methods found to give good results with quinine. The method described by Ramsden and Lipkin seemed to be the best adapted to our purpose, since in the hands of its authors, it appeared to have made possible the estimation of very small quantities of quinine with an error of not more than 5 %. We met with unexpected difficulties in the application of the method, and, after months of effort, we were forced to the conclusion that a strict adherence to the written description of the method would not give us results to which we could attach any quantitative significance. That description seems to us to err in greatly underestimating the difficulty of extracting quinine from blood: with the volume of fluid and amount of extraction which it indicates we could never recover more than 30 to 40 % of the quinine which we added to samples of blood from a number of different species. We found in it, further, no adequate indication of the extreme care which is necessary to avoid the contamination of the extracted quinine with traces of other substances, which affect the turbidity with Tanret's reagent upon which the final determination depends. By increasing the volume of fluid used in extracting the blood coagulum, and introducing as the result of experience various additional precautions against contamination, we have succeeded in obtaining in clean condition practically constant proportions of the quinine added to samples of blood, corpuscles, plasma or serum. With due allowance for the proportion of quinine which, in each case, is, in our experience, irrecoverable by any amount of extraction, a fairly accurate estimate of distribution can be obtained and the rate of absorption and disappearance of quinine from the circulating blood can be followed. Under such conditions it may be possible to obtain comparative figures of moderate accuracy with the other alkaloids. Our purpose here is to indicate the modifications of the Ramsden and Lipkin technique which we have found it necessary to adopt, and the conclusions as regards the distribution of quinine in blood to which we have been led.

Experimental Details.

Standard Reagents.

1. *Aqueous quinine solution* made by dissolving 0·25 g. of anhydrous quinine base in water containing 7·71 cc. of $N/10$ sulphuric acid (one equi-

valent) plus 0·77 cc. of acid to facilitate solution, and diluting to 250 cc. 1 cc. of final solution contains 1 mg. of quinine base.

2. 1 *in* 100,000 *quinine in ammonium sulphate solution*, made by adding 5 cc. of solution 1 to a saturated solution of pure recrystallised ammonium sulphate and diluting to 500 cc. with the same saturated solution.

Solutions 1 and 2 were kept in the dark as there is a marked deterioration of quinine content of 2 on prolonged exposure to sunlight.

3. 0·6 % *sulphuric acid solution of saturated ammonium sulphate* made by adding 12 g. of sulphuric acid to a saturated solution of pure recrystallised ammonium sulphate and diluting with the same saturated solution to 2000 cc.

The ammonium sulphate used throughout the results recorded below was an unusually pure sample containing only the faintest trace of chloride. We find that commercial ammonium sulphate "recryst" is pure enough for the solutions recorded here, after removal of adventitious matter by filtration.

4. *Pure ether, free from aldehydes* prepared as described by Ramsden and Lipkin.

5. *Pure ammonia solution* prepared by distilling 0·880 ammonia through an all glass apparatus and collecting the gas in distilled water at 0°. This precaution is essential owing to the solvent action of strong ammonia on organic matter, grease, etc. and a separation, moreover, is effected from less volatile tar-bases present in traces in commercial ammonia which might affect the subsequent estimation of quinine.

Method of estimating Quinine in Blood.

This is based on the Ramsden Lipkin process with important modifications which will appear in the sequel.

5 cc. of fresh defibrinated blood were added to 1/20 mg. of quinine in a beaker, well stirred and then laked with about 5 cc. of distilled water. 20 cc. of saturated ammonium sulphate solution containing 0·6 % of sulphuric acid were added and 5 g. of solid pure recrystallised ammonium sulphate. The solution was brought to the boil over a free flame, after a preliminary coagulation of the contents on a boiling water-bath, with constant rotation of the contents and filtered through a Gooch crucible packed with a layer of asbestos, covered with a porcelain filter disc and over the latter a filter paper so as to prevent the coagulum passing into the asbestos. The entire coagulum on the filter paper was returned to the beaker, treated with a second 20 cc. of acidified ammonium sulphate solution, disintegrated and the solution again brought to the boil and filtered as before, the coagulum being in each case well pressed down. The same process was repeated ten times in all, the entire coagulum being each time returned to the beaker. The combined filtrate, about 200 cc., was transferred to a well-stoppered 250 cc. measuring cylinder, and 10 cc. of pure ammonia solution added, followed by 20 cc. of pure ether. On shaking with further successive quantities of 15, 15 and 10 cc. of ether, practically the whole

of the quinine present passed into the ether, which was in each case pipetted off by means of a 1 cc. pipette fitted with a rubber teat, and transferred to a boiling tube dipping into a gently boiling water-bath. When every trace of ether had boiled away the quinine was left as a clear colourless varnish which was dissolved in 10 cc. of pure saturated ammonium sulphate solution by just bringing to the boil. The solution was rapidly cooled in water and 5 cc. transferred to one of a series of standard tubes, the remainder of which contained 5 cc. each of various standard solutions of quinine made by mixing the requisite quantities of 1 in 100,000 quinine in saturated ammonium sulphate solution with saturated ammonium sulphate solution. 0·05 cc. of Tanret's reagent was rapidly added to each and the contents mixed. The whole series of tubes was transferred simultaneously to a boiling brine-bath for a few minutes and then rapidly cooled in running water. By comparing the turbidities in uniform daylight (not direct sunlight) against a dark background, the standard solutions differing in strength by as little as 7 % can be invariably placed in the correct sequence of strengths and the solution of unknown strength is also placed without difficulty and is qualitatively indistinguishable from the standards. We have tried the Ramsden nephelometer but in our opinion it fails to offer any advantage over direct observation in daylight.

The points of importance[1], which Ramsden and co-workers have not laid stress on, are that the most scrupulous care must be exercised throughout the experiment so as to ensure complete freedom from all traces of grease of all surfaces with which the solutions, whose turbidities are going to be compared, come in contact. The standard tubes cannot even be washed out with organic solvents to accelerate drying, but must be washed with several changes of distilled water and then dried by draining or by blowing out in a current of warm air.

Mere contact of the final solutions with the fingers is sufficient to vitiate the results and in addition there is the importance of the use of pure ammonia referred to above. Failure to take these precautions causes the turbidities to be qualitatively abnormal and renders quantitative estimation impossible.

Results with the Method.

Using the method as just described the following table shows to what extent quinine can be extracted from blood or its constituents.

	Treatment	Volume cc.	Quinine added mg.	Quinine recovered %	Mean %
Serum		5	0·05	(*a*) 84, (*b*) 84	84
Corpuscles	Laked	5	0·05	(*a*) 77, (*b*) 69	73
Whole blood	Not laked	5	0·05	(*a*) 62, (*b*) 69	65
Whole blood	Not laked (15 boilings)	5	0·05	71	—
Whole blood	Laked	5	0·05	(*a*) 74, (*b*) 74	74

[1] Professor Ramsden informs us that, with the exception of the dangers arising from impurities in the ammonia, they were themselves well aware of them.

It is evident that laking the blood materially assists the extraction of quinine and that recovery from corpuscles is more difficult than from serum.

The effect of increasing the number of changes of boiling fluid and of increasing the volume of the fluid is shown by the following table, 5 cc. of freshly defibrinated goat's blood being used in each case and the quinine added dissolved in saturated ammonium sulphate solution.

	No. of boilings	No. of changes of fluid	Total volume of fluid cc.	Quinine recovered %
(*a*)	10	4	50	44
(*b*)	10	10	100	69
(*c*)	10	10	200	87

That the chief loss lies in the difficulty of extracting the quinine from the coagulum is shown by omitting the blood and adding the quinine to the acidified ammonium sulphate, making alkaline and extracting as described for the remainder of the process. A duplicate estimation was made giving the same result in both. Out of 0·05 mg. added to 200 cc. of acidified saturated ammonium sulphate solution, the quantity recovered lay between 0·05 mg. and 0·045 mg. As an approximation we put it at 95 % recovery.

The substitution of 0·6 % sulphuric acid by weight by 2 % sulphuric acid by volume, or the substitution of saturated ammonium sulphate by 30 % ammonium sulphate in the boiling fluid[1], made no difference to the amount of quinine extracted, as is shown by a comparison of the following table with the first table.

	Volume cc.	H_2SO_4 %	$(NH_4)_2SO_4$ %	Quinine added mg.	Quinine recovered %
Blood	5	2	30	0·05	66
Serum	5	2	Saturated	0·05	80
Corpuscles	5	2	Saturated	0·05	67 and 70

Distribution of Quinine between Serum and Corpuscles.

The distribution of quinine as found by us is shown by the following:

1. 0·10 mg. of quinine was added to 10 cc. of fresh defibrinated goat's blood of which the serum-corpuscle ratio as determined in duplicate was 65/35 by volume.

Quinine found in total serum	0·045 mg.
Quinine found in laked corpuscles	0·027 mg.
Quinine recovered	0·072 mg.

whence it can be calculated that for equal volumes of the two fluids

$$\frac{\text{Quinine in corpuscles}}{\text{Quinine in serum}} = \frac{53}{47}.$$

2. 0·10 mg. of quinine was added to 17 cc. of fresh defibrinated sheep's blood of which the serum-corpuscle volume ratio had been artificially adjusted by removal of serum at 50/50.

[1] These modifications were suggested to us by Professor Ramsden as being more expeditious.

Quinine found in total serum	0·041 mg.
Quinine found in laked corpuscles	0·035 mg.
Quinine recovered	0·076 mg.

whence $$\frac{\text{Quinine in corpuscles}}{\text{Quinine in serum}} = \frac{46}{54}.$$

3. 0·17 mg. of quinine was added to 14 cc. of fresh defibrinated goat's blood of which the serum-corpuscle volume ratio had been artificially adjusted by removal of serum at 50/50.

Quinine found in total serum	0·047 mg.
Quinine found in laked corpuscles	0·043 mg.
Quinine recovered	0·090 mg.

whence $$\frac{\text{Quinine in corpuscles}}{\text{Quinine in serum}} = \frac{48}{52}.$$

Bearing in mind the greater difficulty of extracting quinine from laked corpuscles than from serum, and the impossibility of completely removing serum from corpuscles, these three experiments point to an approximately uniform concentration of quinine in corpuscles and serum.

Quinine and Hydroquinine Content of Human Blood.

One of us took 1 g. of quinine base and on another occasion 1 g. of hydroquinine base (both as bisulphate in aqueous solution) by mouth at 10 a.m. 5 cc. of blood were removed at hourly intervals and the quinine or hydroquinine content estimated. Previous experience with laked blood has shown that as a rule about three-quarters only of the alkaloid can be estimated. By calculating on the basis of a 75 % recovery of quinine from blood the following concentrations of quinine and hydroquinine were found, and if the total volume of blood be put at 5 litres, the percentage of the quinine taken orally which is subsequently found in the blood is determined.

Interval after administration hours	Concentration of quinine in blood	Amount in blood mg.	% of dose taken
1	1 in 150,000	33	3·3
2	1 in 187,000	27	2·7
3	1 in 225,000	22	2·2
For Hydroquinine.			
1	1 in 250,000	20	2·0
3	1 in 280,000	18	1·8

As a point of interest it can readily be shown by calculating from these figures that at the end of one hour the number of molecules of quinine per corpuscle is about 20 millions.

The concentrations reached in the blood after oral administration of smaller doses of quinine are shown in the following table:

Dose	Concentration after 1 hour	Concentration after 2 hours
0·5 g.	1 in 375,000	1 in 500,000
0·25	Below 1 in 375,000	Below 1 in 750,000

As a practical method for estimating traces of quinine in blood we cannot recommend the process. Apart from the non-quantitative recovery of the quinine, a single complete estimation takes three to four hours, the scrupulous care required to be exercised over this period involving a considerable strain on the operator.

In conclusion our results show that neither the preponderance of quinine in corpuscles as maintained by Morgenroth nor the preponderance in plasma as held by Ramsden and Lipkin can be correct. On the contrary, like other observers, we find approximately equal or uniform distribution of quinine between corpuscles and serum. On such evidence it is clearly undesirable to found any theory of the mode of action of quinine in malaria, such as that propounded by Morgenroth.

REFERENCES.

Acton (1920). *Lancet*, June 12th.
Halberkann (1919). *Biochem. Zeitsch.* **95**, 24.
Hartmann and Zila (1918). *Arch. expt. Path. Pharm.* **83**, 221.
Lipkin (1919). *Ann. Trop. Med. Parasit.* **13**, 149.
Morgenroth (1918). *Deutsch. med. Woch.* **44**, 961, 988.
Ramsden (1920). *Brit. Med. J.* ii, 117.
Ramsden and Lipkin (1918). *Ann. Trop. Med. Parasit.* **9**, 443.
Ramsden, Lipkin and Whitley (1918). *Ann. Trop. Med. Parasit.* **12**, 223.

VIII. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS.

PART I.

THE ISOLATION OF PROTEINS FROM LEAVES

By ALBERT CHARLES CHIBNALL and SAMUEL BARNETT SCHRYVER.

From the Biochemical Department, Imperial College of Science and Technology.

(*Received December 8th, 1920.*)

In spite of the importance of this subject, very little is known up to the present about the nitrogenous metabolism of the higher plants. This is due, to a great extent, to the extraordinary difficulty in the chemical technique. A large number of metabolic processes are continually taking place within the leaves, and many different substances must be present at the same time, for the separation of which no well-defined chemical methods have been evolved. The first object of these investigations was the elaboration of methods by means of which the chief nitrogenous constituents of the leaves could be isolated and identified.

The work was commenced in 1916 by one of the present authors (S. B. S.) in collaboration with Miss Laura M. Saunders, but had to be abandoned after a few months work owing to the pressure of other duties. Certain work of a preliminary nature was however brought to a conclusion, and the results obtained by Miss Saunders were utilised when the work was resumed by the present authors at the beginning of 1920.

The first subjects chosen for investigation were cabbages, not because they are ideal for the purpose, but because they could be readily obtained in quantity in London almost throughout the year. The experiments were directed, in the first instance, towards the discovery of a method by means of which the maximum amount of nitrogenous material could be removed from the leaves in the form either of true or colloidal solution. They were treated with water containing various cytolytic agents, and the effect of each was quantitatively investigated. An account of the results obtained is given in the following [see Chibnall and Schryver, 1920]. The most satisfactory agent was ether, and water saturated with ether was found to remove not far short of twice the amount of nitrogen which was dissolved out by water alone.

By treating minced leaves for a short period with water saturated with ether (five parts of leaves to four parts of the ether-water) and then subjecting the mixture enveloped in filter cloth to pressure in a tincture press, a green opalescent fluid was obtained, from which no particles separated on pouring through a folded filter-paper. This fluid however deposited a flocculent precipitate on keeping, very slowly in cold weather, but more readily in summer. On warming gently (to about 40°) a dense flocculent precipitate was formed almost immediately, which was found to consist chiefly of complex nitrogenous compounds. A method was hereby revealed for preparing in mass the proteins of the leaves, which has not been hitherto described. Only when a relatively large quantity of material was in hand appeared a paper [Osborne and Wakeman, 1920], in which a method of preparing proteins from spinach was described, and this must therefore be regarded as the first record of the preparation in mass of the proteins of the leaves. The method employed differs in certain important particulars from that described by the authors of the present paper, who, by applying their process to spinach, have obtained results not altogether in agreement with those of the American authors—the most notable difference being that of the nitrogen content of the products isolated.

The results obtained with cabbage leaves pointed to the possibility of obtaining a general method for isolating in mass a part, at any rate, of the proteins of leaves, and the more detailed examination of the simpler nitrogenous substances in the cabbage was relinquished for the moment, in order to ascertain whether the method for preparing proteins, as outlined above, was of general application to all leaves. In the meantime the investigation of the simpler nitrogenous products of the leaves has been undertaken by Mr H. W. Buston and Mr A. E. D. Burdett, some of whose results are utilised in the present paper.

The method of extracting the proteins by means of ether-water has been applied, in addition to the cabbage, to the leaves of Virginia creeper, grass, scarlet runners and spinach.

In the case of Virginia creeper, the leaves of which were collected just as they were about to change colour, and when active metabolism was apparently finished, no appreciable amount of protein was obtained. From the grass, proteins were separated, but they were obtained only in small quantities, and considerably contaminated with starch. From the leaves of the scarlet runner and spinach, on the other hand, proteins were obtained in fairly considerable quantities. Samples were prepared from two pickings of the former leaves, the earlier when the pods were in active process of formation, and the latter when they were fully formed, and when the general metabolism of the plant was on the down grade. The leaves of the earlier picking yielded a fair quantity of protein, the ether-water extract of the plant being converted into a semi-solid mass on warming, whereas those of the later picking gave a very much smaller yield. These results indicate that the proteins,

which can be isolated by means of ether-water, are obtained in the largest quantity when the plant is in an active state of metabolism, and the amount is likely to have some considerable physiological significance, and to represent the proteins which are in process of active translocation.

By means of ether-water a little less than two-thirds of the total nitrogen in the leaves has been removed. An examination of the residual nitrogen has not yet been made, but it forms part of the programme of subsequent research.

As already indicated, the main object of the present communication has been to give a general account of the method for isolating the proteins of plants, and none of the products obtained has as yet been subjected to exhaustive examination. It is not possible therefore at the present moment to give anything more than a very superficial account of their properties. Work is, however, proceeding on the 150 g. of the crude cabbage product prepared earlier in the year.

The proteins isolated appear to have a nitrogen content in the neighbourhood of 13 %, and that isolated from the cabbage has distinct acidic properties, dissolving in weak alkalis, and being precipitable from this solution by acids; this precipitate does not redissolve on addition of excess of acid.

When the general character of the proteins is considered, it will be seen that theoretically there can exist either distinctly alkaline or distinctly acid products, in addition to those that have the iso-electric point only slightly on either side of neutrality. The alkaline proteins should yield on hydrolysis preponderating amounts of di-amino-acids. The protamines, isolated from fish sperm, belong to this class. Acid proteins on the other hand should yield on hydrolysis large amounts of dicarboxylic acids (glutamic and aspartic acids). Proteins of this class have, so far, not been described. The plant globulins, however, on hydrolysis yield large amounts of dicarboxylic acids, but they are not distinctly acidic in character in that they also yield large amounts of ammonia, corresponding roughly to the excess of the carboxyl groups over the amino groups in the molecule. This indicates that these proteins have been isolated in the form of amides. Now it is worthy of note that the protein of cabbage which has been isolated is acidic in properties, and yields on hydrolysis an amount of ammonia (amino-nitrogen) corresponding to 5 % of the total nitrogen, which is distinctly below that of the other plant proteins. These results indicate that acidic proteins exist in the leaves, which, when they pass into the resting stage in the seed, undergo amidisation. Such proteins should contain a relatively small amount of nitrogen. Whether the low percentage found in the samples described in this paper is due to this cause or whether they are conjugated with some nitrogen-poor prosthetic group can only be determined by subsequent research.

There exists in the leaves an appreciable amount of ammonium salts, and the study of the equilibrium of these, the amino-acids, and the proteins, should throw considerable light on the nitrogenous metabolism of the plants.

The proteins from scarlet runner leaves yield an appreciably higher number

for the amide nitrogen than the cabbage protein. It is conceivable that the leaf proteins can exist more or less completely amidised in different stages of the development of the plants, and it is hoped that at an appropriate season opportunity will be offered for investigating this point. It may account for the difference in the properties of the proteins already isolated by the ether-water method. In the meanwhile, material obtained during the summer months is under examination, and the results will, it is hoped, form the subject of a later communication.

The rather full analytical details given for the cabbage are quoted as an illustration of the methods employed rather than as accurate totals, which must of necessity be incomplete until the nitrogen in the residue has been more fully investigated.

Experimental.

Preliminary experiments on cabbage leaves.

A preliminary series of experiments was made in 1916 by Miss Laura M. Saunders under the direction of one of the present authors (S. B. S.). The soft parts of the cabbage were minced, and treated with various solvents, as enumerated below, and the total nitrogen thereby passing into solution was estimated by Kjeldahl's method. Table I gives the results obtained. In each case the solvent was allowed to act for several hours, and the liquid afterwards expressed through muslin in a tincture press.

Table I. *Showing the effect of various cytolysing agents.*

Weight of cabbage used, 200 g.

Solvent	Weight of N extracted in g.
Water saturated with ether	0·38
Water saturated with chloroform	0·335
M/2 benzoate of sodium	0·259
Water containing 20 g. chloral hydrate per litre	0·304
Distilled water	0·220

This indicated that ether-water was the most efficient agent, whilst further experiment showed that the proportion of five parts by weight of minced cabbage to four of the ether-water was ample, also that 20 minutes was sufficient time to leave the mixture before pressing.

The investigation of the liquid extract was taken up by the present authors in January 1920. A dozen cabbages, purchased in the ordinary market, were taken, the hard ribs and heart removed, and the remainder minced in a mechanical mincer, using a fairly coarse cutting plate. Very little, if any, liquid was expressed during this operation, but the leaves were well torn up and lacerated so as to expose a large surface to the cytolysing agent. They were then mixed with ether-water in the proportions stated above (five by weight of cabbage to four of the water), and after standing for 20 minutes the fluid was expressed through thick muslin in a tincture press. It was

green in colour, opalescent, and filtered slowly, but without leaving a residue, through filter paper at the pump. Attempts were next made to concentrate it under reduced pressure at 40°. It was found however that a green solid separated with rise of temperature, causing such violent frothing that the operation had to be suspended. This indicated that the liquid as it came from the press contained some substance in colloidal solution, and attempts were made to cause this to flocculate before concentration. Standing for several days, either alone, or with the usual flocculating salts, did not bring about the desired effect (the room temperature was about 13°; this will probably account for the difference observed in June, when the room temperature was 25°), whereas warming on a water-bath at the ordinary pressure to 40° caused the immediate formation of a thick precipitate, which settled at once on cooling, leaving a clear solution that could be concentrated quietly without trouble. This gave a method of obtaining the products of the extraction in a concentrated form suitable for storing, and 135 cabbages were forthwith so treated. Each batch of about 12 was extracted twice with the ether-water, the second extract from one batch being used as the first in the next, and so on. In all 70 kilos of minced material were treated, and the resultant clear liquid concentrated to about 10 litres after separation of the colloid.

The green colloidal precipitate. This was washed twice with two litres of 95 % alcohol, then with two litres of 0·730 ether and finally allowed to dry in the open air. (The alcoholic washings on evaporating to dryness and purifying by extraction with warm water gave a dark coloured oil consisting almost entirely of chlorophyll. The ether washings likewise consisted chiefly of pigment, giving a solid on evaporation.) On rubbing lightly through a 0·1 mm. sieve, 290 g. of a fine olive-green powder were obtained. The fine powder was then exhaustively extracted with ether in a Soxhlet, and the resultant product, of a dull green colour, weighed 165 g. It contained 11 % of moisture, and the dry material 10·6 % N and 0·255 % P. The extract from the Soxhlet consisted of fats and lipoids with a small amount of green colouring matter.

The high nitrogen content of this precipitate showed that a large proportion of it was apparently protein. Water extracted a small amount of non-nitrogenous matter, leaving a residue which now contained 11 % of nitrogen. The only solvent found for it was aqueous caustic soda, which first gelatinised, and then slowly dissolved a little more than half of it. The strength of the soda used was 0·3 %, and three extractions were made. The residue was centrifuged off, washed with graded strengths of alcohol, then ether, and dried in an oven at 108°. It contained 8·85 % of nitrogen. The alkaline solution remained clear on exact neutralisation, but gave an immediate precipitate on being made slightly acid. This was centrifuged off, washed with graded strengths of alcohol, then ether, and dried in the air. It contained 11 % of nitrogen. On redissolving in soda an appreciable amount was found to be insoluble. After this had been centrifuged off the clear liquid

was again made acid, and the resultant precipitate was found to contain 12·8 % N and a trace only of phosphorus. It was now completely soluble in soda, and could be reprecipitated unchanged. It gave a strong biuret reaction and was new in character in that it was insoluble in excess of acid. Table II shows the distribution of the nitrogen in this compound, as determined by Osborne's modification of Haussmann's method.

Table II. *Distribution of nitrogen in the cabbage leaf protein prepared in the winter.*

	% N.
Amide nitrogen ...	5·89
Humin „ ...	5·23
Basic „ ...	21·95
Mono-amino nitrogen (by diff.) ...	66·93

The clear mother liquid. The 10 litres that had been prepared were poured into an equal volume of 95 % alcohol, when a copious white precipitate was formed, which settled on standing. The supernatant liquid was siphoned off and the residue sucked dry at the filter-pump, washed with alcohol, then ether, and dried in the air. The clear alcoholic liquid was again concentrated under reduced pressure, and has been stored since under toluene and a little chloroform.

Mr H. W. Buston is at present at work on the isolation of the individual amino-acids in this liquid. He has shown that 9·49 % of the total nitrogen is in the form of ammonium salt, and after the removal of this, 25·1 % in the form of amino-nitrogen. Also that 20 % of the total nitrogen is precipitated by means of lead salts. After the removal of this the major part of the residual nitrogen is precipitable by phosphotungstic acid.

The alcoholic precipitate weighed 300 g., and was white when dry. On analysis it gave 6·5 % of moisture, and the dry substance 1·90 % of nitrogen and 1·64 % of phosphorus.

Part of it was extremely soluble in water, and could be purified by repeated reprecipitation in 80 % alcohol. Nothing further has been done to identify this substance. Several grams of it have been prepared, which give a colourless solution in water. A similar sample prepared by Miss Saunders in 1916 gave the following analysis: Ca, 24 %; SO_4, 18 %; P, 1 %. It probably contains free calcium sulphate.

Of the residue insoluble in water, part was soluble in 5 % acetic acid. It could be precipitated by alcohol, and was purified by repeated solution and reprecipitation. It gave an ash weight of 65 %, and the following analysis (Saunders): Ca, 22·5 %; P, 11·7 %; C, 12·2 %; H, 2·35 %. No nitrogen and a trace of halogen. Molisch's test was positive on standing. It is probably some conjugated calcium or calcium-magnesium phosphate, but does not appear to be phytin in that it is not precipitated from acetic acid solution by boiling. The material will be investigated later.

Analysis of the Protein of the Cabbage Extract.

Having made this brief survey of the various products that had thus been prepared, it was decided that, for the present, attention should be given to the substance that obviously contained much protein. To obtain accurate data as to its percentage weight in the cabbage, and the proportion of the total nitrogen that it contained, a quantitative analysis was made of another sample of six cabbages, purchased early in June. These were a little different from those used earlier in the year. They were smaller; both the inner and outer leaves appeared to contain more green colouring matter; and the area of small yellow leaves, growing around the heart, was much less.

As before, the heart and thick fibrous stems were removed, and the remainder minced. The weight of the minced material was 1877 g., and of this 297 g. (leaving 1580 g.) were placed in an oven heated to 108° for the purpose of obtaining the dry weight and the total nitrogen. From these data it was estimated that the 1580 g. used for the experiment contained 157·7 g. of solid matter; 6·18 g. N; and 25·4 g. of ash.

This 1580 g. was then thoroughly mixed with 1200 cc. of ether-water, allowed to stand for 20 minutes, and then pressed out through thick muslin in the tincture press. The total volume of the extract was 2440 cc. The residue, removed from the filter-cloth, was again passed through the mincer, and then mixed with another 1000 cc. of the ether-water, allowed to stand for 20 minutes, and pressed out. The volume of this extract was 965 cc. The residue was then again removed from the filter-cloth, crumbled up by rubbing between the hands, and treated successively with another 1000 cc. of the ether-water three times, the volumes of the extracts being 935, 940 and 940 cc. respectively.

The final residue, after the fifth pressing, was dried in an air oven at 108°. It weighed 67·5 g. and contained 2·32 g. N.

The nitrogen in the several extracts was also determined, the results being given in Table III. It will be observed that the first extract contains 80·9 %, and the first with second combined, 90·2 % of the extractable nitrogen.

To test if any of the nitrogen was held back in the pores of the filter-cloth, the latter was washed first with water and then three times with 80 % alcohol. A quantity of green colouring matter was removed, but the total nitrogen in all the washings was less than 0·05 g.

The green colloidal precipitate. It was not found necessary to heat the extracts so obtained to coagulate the protein material, as the temperature at which the experiment was being conducted, 22–25°, was sufficient to cause a heavy precipitate to come down during the course of the night, leaving an almost clear solution.

The precipitate from the first extract, called "A" henceforth, was separated by decanting and centrifuging, as was also that from the other four solutions combined, called henceforth "B."

Table III. *Showing the distribution of N after extracting five times with ether-water.*

	Total volume or weight	Weight of N in g.
1st ether-water extract	2440 cc.	3·066
2nd „ „	965 „	0·352
3rd „ „	935 „	0·191
4th „ „	940 „	0·096
5th „ „	940 „	0·088
Total extract	6180 „	3·793
Dry residue	67·5 g.	2·320
Total nitrogen	—	6·113
Taken for experiment (estimated)	—	6·18

Table IV. *Showing total solids precipitated by standing 24 hours (after washing with alcohol and ether).*

	Weight in g. moisture free	Weight of N in g.	% of N
Precipitate A	3·70	0·452	12·1
„ B	0·448	0·052	11·9

Both A and B were washed with 80 % alcohol until all the chlorophyll was removed, then three times with 0·730 methylated ether, and left to dry at the room temperature.

Table IV summarises these weights in terms of the total cabbage taken.

The alcoholic washings of A and B consisted almost entirely of chlorophyll, with a certain small amount of the soluble nitrogenous substances carried down during the precipitation. The dry weight was 1·05 g., and the nitrogen content 0·056 g.

The ether washings of A and B on evaporation gave an orange-yellow grease, weighing 0·18 g.

The original extracts, after the precipitation of A and B, were still slightly cloudy. It was found that raising the temperature to boiling point caused another slight coagulation to occur, and a deposit, called henceforth C, to settle during the course of the night. After this had been removed by centrifuging, the nitrogen in each solution was again determined. All five were then mixed, and an aliquot part evaporated to dryness and heated to 108° in an oven. From this it was calculated that the weight of the water-soluble material was 73·1 g.

The coagulate C, the major portion of which was thrown down from the first extract, was washed with water, which removed 0·392 g. of material containing 0·021 g. N, then with alcohol, which removed a further 0·159 g. of material, containing less than 0·01 g. N and finally with ether, being allowed to dry off at room temperature. The weight, moisture free, and calculated for the total cabbage taken, was 0·608 g., and it contained 0·074 g. N.

The results of the preceding analyses are summarised in Table V.

Table V. *Showing distribution of nitrogen.*

	Weight in g. moisture free	Weight of N in g.
1st clear solution	73·1 of solid matter	2·370
2nd „ „		0·312
3rd „ „		0·165
4th „ „		0·076
5th „ „		0·078
Precipitate A	3·74	0·452
„ B	0·45	0·054
Coagulate C	0·68	0·074
Alcoholic washing of A and B	1·05	0·056
Ether „ „	0·18	?
Water washing of C	0·39	0·021
Alcoholic washing of C	0·16	?
Residue	67·50	2·32
Total	147·25	5·992
Taken for experiment (estimated)	157·7	6·18

Examination of precipitates A *and* B.

These were mixed and treated with 0·4 % caustic soda. Considerably more than half was found to go into solution this time, imparting to it as before a deep brown colour. The residue was centrifuged off and treated twice again in the same way. The third solution on being made acid gave very little precipitate, so it was resolved to treat with hot alcoholic alkali according to the method described by Osborne and Wakeman [1920] in their paper on spinach leaves, which had come into our hands about this time. The first application of 0·3 % potash in 60 % alcohol dissolved about half the residue, and gave quite a heavy precipitate on being made acid. A second application dissolved practically nothing. The residue, after being thus treated, was washed with graded strengths of alcohol, then ether, and dried at 108°. It weighed 0·136 g. and contained only 0·003 g. N.

The aqueous soda extract was made slightly acid, and the heavy precipitate allowed to settle. The supernatant liquid was poured off, and the remainder centrifuged. The solid was redissolved in the same strength soda. It was again precipitated with acid, centrifuged, washed with graded strengths of alcohol, then ether, and allowed to dry at the room temperature. The dried product weighed, moisture free, 2·247 g. and it contained 12·81 % N, and only a trace of phosphorus.

The residual acid solution, together with the alcoholic washings, was found to contain 0·129 g. of nitrogen.

The alcoholic soda extract was treated in a similar way. The final product weighed, moisture free, 0·162 g., and it contained 12·7 % N. The weight of nitrogen remaining in this solution and the washings was 0·005 g. Table VI shows the complete analysis of the precipitates A and B, calculated for the

total weight of cabbage taken. It will be seen that 63·3 % of the total weight of A and B is recovered from the aqueous soda solution, and 4·55 % from the alcoholic; in all 67·85 %. Of the total weight going into solution, 70·6 % is reprecipitated.

Table VI. *Showing the extraction of precipitates* A *and* B *by aqueous and alcoholic soda.*

	Weight in g.	Weight of N in g.
Protein, precipitated from aqueous soda	2·651	0·339
„ „ „ alcoholic soda	0·191	0·024
Non-protein substances, soluble in aqueous soda and acid	1·185 (by diff.)	0·139
„ „ „ alcoholic soda and acid		0·005
Insoluble residue	0·161	0·003
Total ...	4·188	0·516

Table VII. *Showing the distribution of nitrogen in the cabbage leaf protein prepared in summer.*

	% N
Amide nitrogen	5·62
Humin nitrogen	5·15
Basic nitrogen	21·31
Mono-amino nitrogen	67·92

The figure 12·8 for the % N in the final product is in agreement with that obtained for the similarly prepared compound from the cabbages purchased several months earlier. The internal distribution is also very similar, as will be seen from Table VII.

Table VIII shows the results of the complete analysis.

Table VIII. *Final results of the analysis.*

	Weight in g. moisture free	% weight, moisture free	Weight of N in g.	% N
In aqueous solution	73·49	46·59	3·02	48·87
In colloidal suspension in water:				
Protein	2·84	1·80	0·36	5·83
Non-protein substances	1·19	0·76	0·13	2·13
Albumin (? coagulated on boiling)	0·68	0·43	0·07	1·13
Soluble in alcohol and ether ...	1·39	0·88	0·07	1·13
Residue	67·66	42·80	2·32	37·64
Total ...	147·35	93·26	5·97	96·73
Taken for analysis (estimated) ...	157·70	—	6·18	—

Cut Grass.

Short lengths of grass, as cut by a lawn-mower, were obtained from the Botanical Gardens, Chelsea. After being washed and air dried they were minced, with a fine cutter, and treated with ether-water as before. 1420 g. of minced material were used, and from experiments on an aliquot part it

was estimated that this amount contained 857 g. of solid matter, or 60·4 %, and 32·8 g. of nitrogen.

The amounts of nitrogen expressed in the five ether-water dressings are given in Table IX.

Table IX. *Showing the weights of N in the ether-water extracts from grass.*

	Weight of N in g.
1st extract	4·79
2nd „	2·35
3rd „	1·36
4th „	0·79
5th „	0·66
Total of 5 extracts	9·95

The solution from the press was olive-green as in the cabbage, and gave no deposit on prolonged centrifuging. Experiments to bring about flocculation were then tried. Standing for two days, either unchanged or after warming to 60°, failed to bring this about. The only reagents successful were alcohol in the proportion of 1 in 4 and mineral acid.

As the cabbage had given a final product that was precipitated from solution by acids, it was resolved to use this method in the present case. It was found that the addition of enough sulphuric acid to make the whole $N/12$ was sufficient to give a definite precipitate and leave a clear solution. Acid of this strength also would be insufficient to bring about any hydrolysis in the cold. The material so obtained was washed with alcohol and ether as before and air dried. It contained 6 % N only, but was soluble in 0·3 % aqueous soda from which it could be reprecipitated by acid. The product so obtained contained 8·25 % N. It failed to give any of the colour reactions for a protein, and as the amount obtained was small and we were desirous of investigating other leaves before the season became too late, nothing further was done with it.

Scarlet Runner Leaves.

These were picked at the end of the first week in September, when the beans were still in process of formation. They were cut so as to leave no stem, and after being cleansed were minced, using a coarse cutting plate. They appeared to undergo very considerable disintegration, the minced product being a dark green pulp, containing a large quantity of expressed juice.

The total weight minced was 1331 g. and of this 110 g. were taken for dry-weight analysis. From the result of this it was estimated that the 1221 g. used in the experiment contained 185·8 g. of solids, or 15·2 %, and 8·21 g. N.

It was then treated five times successively with ether-water, being re-minced before the second application. 1000 cc. were used in each case, and the results are given in Table X.

Table X. *Showing distribution of nitrogen in bean leaves after extraction in the ether-water.*

	Weight of N in g.
1st extract	4·07
2nd „	0·75
3rd „	0·37
4th „	0·16
5th „	0·16
Total in 5 extracts	5·51
In the residue, weight 79 g. ...	2·60
Total ...	8·11
Taken for experiments (estimated)	8·21

In comparison with the cabbage it will be seen that the total nitrogen expressed in the first extract is about the same, *i.e.* nearly one-half; the residue similar, being 42 %, and the total expressed nitrogen greater, being 68·4 % as compared with 62·4 %.

All the extracts, especially the first, were deep green and opaque. A sample of the latter, on warming to 40°, flocculated into a solid mass, and the main bulk was diluted with an equal volume of water before being heated. The precipitate, called henceforth D, after washing with alcohol and ether as described above had a moisture-free weight of 15·5 g., and contained 10·3 % N.

The similar product from the second and third extracts, called henceforth E, weighed, moisture free, 2·2 g. and contained 8·5 % N.

Extracts 4 and 5 failed to flocculate on warming to 40°, but warming to 60° brought this about. This temperature also caused a second flocculation in the first three extracts, and the combined products, called henceforth F, after washing as before, weighed, moisture free, 4·7 g. and contained 10·8 % N.

All the extract solutions were now a clear light brown. The first on boiling gave a third small coagulate, but the others remained clear. This coagulate weighed 0·52 g., contained 9·1 % N and is probably the impure product from some water-soluble albumin. It appeared to be insoluble in all common solvents.

Table XI shows the results of the foregoing precipitation after allowance has been made for the quantities withdrawn for experiment.

Table XI. *Showing the products obtained by warming the ether-water extract of bean leaves.*

	Weight in g.	% N	g. N
Precipitate D ...	16·5	10·3	1·70
„ E ...	2·3	8·5	0·19
„ F ...	5·0	10·9	0·55
Coagulate on boiling	0·5	9·1	0·05
Total weight ...	24·3	—	2·49

It will be seen from this that 13·1 % of the total dry weight is precipitated in the form of a highly nitrogenous body containing 38·4 % of the total nitrogen.

Examination of Precipitates D, E *and* F.

Precipitate D was found to contain starch, and was freed from this by repeated washing with water at 60° until the washings gave no reaction with iodine solution. The resultant product was found to be almost insoluble in cold aqueous soda (0·3 %), but went up readily in alcoholic potash (0·3 % in 60 % alcohol) warmed up just to the boil on a water-bath. After centrifuging, it was just made acid with dilute sulphuric acid, when a copious precipitate came out, which, unlike the cabbage product, was soluble in excess. It was now partially soluble in cold aqueous soda. The remainder was taken up again in hot alcoholic potash, and on reprecipitation was found to be completely soluble in cold soda. The combined solutions in cold soda were centrifuged, yielding a clear liquid, which threw down a heavy white precipitate on being made just acid. This was centrifuged off, washed with alcohol and ether as before, and dried in the air. The product weighed, moisture free, 5·88 g., and contained 13·33 % N, and very small traces of phosphorus. It would appear (from its mode of preparation and properties) to be of the nature of an alkali albumin.

Precipitate E was worked up in the same way, starch being first removed. As before hot alcoholic potash was required, the product after two washings becoming soluble in cold soda. Whilst drying it was accidentally lost by the cracking of the beaker, but the yield appeared to be the same as in the other two preparations, *i.e.* about 40 %.

Precipitate F contained no starch, which probably accounts for its higher percentage of nitrogen. As before hot alcoholic potash was required to dissolve it, the product obtained after acidification becoming soluble in cold soda after a second washing. Washed and dried the final preparation weighed, moisture free, 2·15 g., and contained 13·4 % N.

The resultant products from D and F are probably the same, for they behave in exactly the same way with acids and alkalies, give a strong biuret reaction, and contain about 13·5 % N.

Table XII gives the distribution of the nitrogen in the product from D.

Table XII. *Showing the distribution of nitrogen in the bean leaf protein.*

	% N
Amide nitrogen	9·38
Humin nitrogen	7·95
Basic nitrogen	28·66
Mono-amino nitrogen	54·01

It will be observed that the amide nitrogen is considerably higher than in the case of the cabbage product, possibly accounting for its less acidic character.

The working up of this bean material was not done with quite the same care as that bestowed on the cabbage, so that small losses may have been entailed. Allowing for this, and calculating the product to be obtained from

E as 40 % of its weight, we estimate the total yield of this protein to be 9 g., containing 1·12 g. N. Expressed as percentages, 5 % of the dry weight and 13·8 % of the nitrogen are isolated in the form of a protein.

Considering that the combined weight of D, E and F was nearly 24 g., this yield is very low. A comparison of it with that of the cabbage and spinach (Osborne's figures) is given in Table XIII.

Table XIII. *Showing the different effects of aqueous and hot alcoholic alkali on the proteins of the cabbage, bean and spinach leaves.*

	% weight recovered		
	Cabbage	Bean (mean)	Spinach (Osborne)
From cold aqueous soda	63·3	—	58·6
From hot alcoholic potash	4·6	38	13·4
Total ...	67·9	38	72·0

These figures are instructive in that they show that a high yield is obtained only when the reaction takes place in cold aqueous soda. It seems probable therefore that warming with alcoholic potash to the boil brings about a greater degree of hydrolysis than we at first suspected, the solution containing degradation products that are not precipitated in slight excess of acid. With regard to this question of the degree of solubility in cold soda, we have been struck by the way in which this property seems to vary in the different preparations from the same plant. From the experience gained in working up the various products described in this paper we have come to the conclusion that the previous treatment the first precipitate undergoes in its washing with alcohol has something to do with it. In one case in particular, in which some of the precipitate from spinach was left over the week-end in absolute alcohol, we found the resultant product, dried by ether, to be quite insoluble in the cold. Possibly strong alcohol has some denaturing effect on the protein present.

Spinach.

About a kilo. of spinach was worked up at the beginning of October by our method in order to compare the final product obtained with that isolated by Osborne and Wakeman [1920]. Only 57 % of the total nitrogen passed into solution, which was divided into two parts. One was warmed to 40° to flocculate the colloid by the method described above, the other was flocculated by adding a quarter volume of alcohol, as described by Osborne. In both cases the washed and dried product gave about 10·4 % N, showing that the two methods had apparently the same result. On extracting with cold soda and precipitating with acid a protein-like substance was isolated which gave only 12·6 % of nitrogen and was insoluble in excess of acid. It appeared therefore to be a substance with an acidic character similar to that isolated from the cabbage.

This result does not correspond at all with that of Osborne and Wakeman. Their method is first to grind the fresh leaves three times in a Nixtamal mill, then add water in the proportion of 1 litre to 1 kilo. of spinach, and regrind the mixture twice. They then centrifuge to remove the disintegrated cellular matter and obtain a green opaque solution containing a large quantity of protein in colloidal suspension. They flocculate as described above by adding alcohol, and after washing and drying obtain a substance averaging 14 % N. This substance they then treat in the same way as we do, *i.e.* with cold soda, and on making the solution slightly acid bring down a substance giving 15·3 % of nitrogen. This differed from all our preparations in that it was soluble in slight excess of both acid and alkali.

A chemical explanation of this difference we are at present unable to give, but it may be a purely physiological one. From the very large proportion of the total nitrogen that they actually isolate as protein (43 %), it would appear that they took their leaves at a period of great physiological activity. They do not give the time of year when their leaves were picked, nor the period between picking and working up. In our case the leaves were purchased in a shop, and were probably picked two days previously. This period may have allowed some enzyme action to have taken place, but if that were the case we should have expected a much higher nitrogen percentage in the protein of the bean leaves, which were treated the same day that they were picked. The explanation seems to us, in view of the small amount of evidence yet available, to be due to different chemical compounds being actually present in the leaves taken.

For comparison the distribution of the nitrogen found by them in their compound is given in Table XIV.

Table XIV. *Showing the distribution of nitrogen in the spinach leaf protein* (Osborne and Wakeman).

	% N
Amide nitrogen	7·50
Humin nitrogen	5·97
Basic nitrogen	22·49
Mono-amino nitrogen	64·04

SUMMARY.

(1) A method has been described for obtaining the proteins of green leaves.

(2) This consists in treating the disintegrated tissue with water containing a cytolytic agent, for which purpose ether was found to be the most suitable.

(3) By means of water saturated with ether, opalescent colloidal solutions are obtained from which flocculation of the colloid takes place, either on standing, if the room temperature is sufficiently high (above 20)°, or more rapidly on warming to 40°.

(4) The colloidal precipitates obtained in this manner from the leaves of cabbage, scarlet runners and spinach have been examined and found to consist for the most part of proteins, and to contain about 10 % N.

(5) Methods have been described for obtaining the proteins in a purer condition, and products containing about 13 % N have been prepared.

(6) The cabbage and spinach proteins are, when freshly prepared, soluble in weak alkaline solutions from which they can be precipitated by acids, in excess of which they are not speedily soluble. The protein of scarlet runner was insoluble in weak aqueous alkalies.

REFERENCES.

Chibnall and Schryver (1920). *J. Physiol.* **54**, *Proc.* July.
Osborne and Wakeman (1920). *J. Biol. Chem.* **13**, 1.

IX. STUDIES ON THE CYCLOCLASTIC POWER OF BACTERIA.

PART II.

A QUANTITATIVE STUDY OF THE AEROBIC DECOMPOSITION OF TRYPTOPHAN AND TYROSINE BY BACTERIA.

By HAROLD RAISTRICK and ANNE BARBARA CLARK.

From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Council.

(*Received December 17th, 1920.*)

The method used by Raistrick [1919], in the quantitative study of the decomposition of histidine by bacteria, has been applied with slight modification to the study of the decomposition of the cyclic nuclei of tryptophan and tyrosine. The solution of mineral salts used in these experiments differed slightly however from that previously employed and had the following composition recommended for the purpose by Koser and Rettger [1919]:

Distilled water	1000 cc.
NaCl	5·0 g.
$MgSO_4$ ...	0·2 g.
$CaCl_2$	0·1 g.
KH_2PO_4 ...	1·0 g.
K_2HPO_4 ...	1·0 g.

I. Experiments on the Decomposition of Tryptophan.

4 g. of tryptophan were dissolved in 2 litres of the foregoing saline solution. In duplicate portions of 75 cc. the total N [A] was estimated by Kjeldahl's method. 200 cc. of the medium were pipetted accurately into each of nine flasks (250 cc. capacity). To each of three of these 2 cc. of glycerol were added. All the flasks were then plugged with cotton wool and autoclaved at 115° for 20 minutes. Of the nine flasks

(*a*) One without glycerol was used for the determination of the distribution of N in the medium without precipitation with colloidal iron.

(*b*) One without glycerol was used for the determination of the distribution of N in the medium, using precipitation with colloidal iron as described later.

(*c*) Four without glycerol were inoculated with pure cultures of one of the following bacteria:

1. *B. fluorescens.*
2. *B. pyocyaneus.*
3. *B. prodigiosus.*
4. *B. proteus vulgaris.*

(*d*) The three flasks containing glycerol were inoculated with pure cultures of one of the following organisms:

1. *B. fluorescens.*
2. *B. pyocyaneus.*
3. *B. prodigiosus.*

The inoculated flasks were then incubated at 37° for a specified number of days.

Analysis of medium for distribution of N.

After incubation (or in the case of the control without incubation) 8 cc. *N* acetic acid were added to the 200 cc. medium. Then 25 cc. of colloidal iron (1·25 %) were introduced drop by drop in the cold with constant shaking. The flask was heated on a water-bath to flocculate the iron precipitate, then cooled, and the contents filtered on a small Buchner funnel containing a filter paper covered with a thin layer of kieselguhr. The residual iron precipitate was washed with hot water, dried in a steam oven, and the nitrogen contained in it was estimated by Kjeldahl's method (synthesised N [E]). The combined filtrate and washings were transferred to a litre Claisen distilling flask, and 100 cc. alcohol and 10 cc. of a 10 % suspension of lime were added. The ammonia which was present was distilled *in vacuo* and absorbed in a known amount of standard acid, the excess of acid being titrated with standard thiosulphate (ammonia N [C]). The residue in the Claisen flask was then made just acid with HCl, evaporated to a volume of a few cc., washed out quantitatively and made up to a volume of 25 cc. The amino N content of this was estimated in the usual way by van Slyke's method (amino-acid N [B]).

In this way the following values, expressed as mg. N per 100 cc. medium, were obtained:

A. Total N.
B. Amino-acid N.
C. Ammonia N.
D. Non-amino N (= A − [B + C + E]).
E. Synthesised N.

From a consideration of these values the following information is obtained:

1. Amino-acid N [B] gives the N contained in the amino-group of the side chain, and hence shows to what extent the amino-group has been broken down.

2. Ammonia N [C] gives the whole of the N, whether originating from the amino-group or from the nucleus, which has been metabolised by the bacteria into ammonia.

3. Non-amino N [D] gives a measure of the degradation of the N contained in the nucleus, and hence a decrease in this indicates that the indole nucleus has been attacked and ruptured.

4. The use of colloidal iron as a precipitant enables one to obtain a measure of the amount of N used for synthesis into complex N compounds. This includes the N contained in the bacterial cell and that in the proteins etc. which may diffuse out of the cell. No amino N arising from the tryptophan is included in the colloidal iron precipitate since it is a well-known fact that colloidal iron does not adsorb amino-acids. This was definitely proved by the comparative experiments, previously mentioned with and without the use of colloidal iron. The results of our experiments are recorded in Table I.

Table I. *Composition of medium in each case expressed as mg. N per* 100 *cc.*

1. *Original Medium*		2. *Original Medium after treatment with colloidal iron.*	
A. Total N ...	26·89	A. Total N	26·89
B. Amino-acid N	14·06	B. Amino-acid N	13·95
C. Ammonia N	0·30	C. Ammonia N	0·22
D. Non-amino N	12·53	D. Non-amino N	12·34
		E. N in colloidal iron precipitate	0·38

Tryptophan + Glycerol					Tryptophan without Glycerol				
Incubation period, days			Increase of N	Decrease of N	Incubation period, days			Increase of N	Decrease of N
				B. pyocyaneus.					
13	Total N ...	26·89	—	—	19	Total N ...	26·89	—	—
	Amino-acid N	3·21	—	10·74		Amino-acid N	0·40	—	13·55
	Ammonia N	0·47	0·25	—		Ammonia N	19·15	18·93	—
	Non-amino N	10·85	—	1·49		Non-amino N	3·00	—	9·34
	Synthesised N	12·36	11·98	—		Synthesised N	4·34	3·96	—
				B. fluorescens.					
16	Total N ...	26·89	—	—	27	Total N ...	26·89	—	—
	Amino-acid N	1·47	—	12·48		Amino-acid N	0·02	—	13·93
	Ammonia N	0·03	—	0·19		Ammonia N	16·57	16·35	—
	Non-amino N	7·19	—	5·15		Non-amino N	2·92	—	9·42
	Synthesised N	18·20	17·82	—		Synthesised N	7·38	7·00	—
				B. prodigiosus.					
18	Total N ...	26·89	—	—	38	Total N ...	26·89	—	—
	Amino-acid N	9·56	—	4·39		Amino-acid N	5·90	—	8·05
	Ammonia N	0·07	—	0·15		Ammonia N	9·84	9·62	—
	Non-amino N	13·26	0·92	—		Non-amino N	10·10	—	2·24
	Synthesised N	4·00	3·62	—		Synthesised N	1·05	0·67	—
				B. proteus vulgaris.					
					33	Total N ...	26·89	—	—
						Amino-acid N	9·15	—	4·80
						Ammonia N	5·19	4·97	—
						Non-amino N	12·05	—	0·29
						Synthesised N	0·50	0·12	—

Discussion of Results.

From a consideration of the results given in Table I, the following conclusions may be drawn:

1. Both in the presence and absence of glycerol, *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus* can attack and produce ammonia from both the side chain and the indole nucleus of the tryptophan molecule.

2. Whereas with *B. pyocyaneus* and *B. fluorescens* ammonia is produced from the nucleus almost as readily as from the side chain, *B. prodigiosus* produces ammonia from the side chain much more quickly than from the nucleus.

3. With *B. proteus vulgaris* ammonia formation is limited to the side chain nitrogen. The failure to open the indole ring is analogous to the inability of the same organism to rupture the iminazole ring [Raistrick, 1919].

4. The effect of the presence of an alternative source of carbon is seen from the results obtained with *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus* in the presence and absence of glycerol. Whereas in the absence of glycerol much free ammonia is produced but relatively small amounts of synthesised N, in the presence of glycerol practically no free ammonia is produced, and in two of the three cases the amount of ammonia is actually less at the end of the experiment than at the beginning. This non-formation of free ammonia is coincident with large increases in synthesised N.

These results offer an explanation for a number of observations, made by various bacteriologists [Kendall and colleagues, 1912–1913] during the last few years, on the so-called "protein-sparing" action of carbohydrates. These observers find that, when bacteria are grown in ordinary peptone media containing no carbohydrate, there is progressive production of ammonia, often in considerable amount. In the presence of an available carbohydrate, however, only quite inappreciable amounts of ammonia are formed, although growth is often better in the latter case than in the former. This failure to produce ammonia from carbohydrate-containing media they ascribe to the "protein-sparing" action of the carbohydrates. The first obvious criticism of this interpretation is the fact that these workers regard ammonia formation as an index of protein decomposition, and make no other attempt to estimate the extent to which protein decomposition has taken place. We consider that the best method of measuring protein and amino-acid decomposition is to follow the disappearance of amino-acid N. Our results show that an amino-acid is decomposed by bacteria in the presence of glycerol without the production of a corresponding amount of ammonia, the N of which appears in the form of synthesised N. It seems to us that the probable explanation of the so-called "protein-sparing" action of carbohydrates is the following. Bacteria need for their anabolic processes certain relative amounts of nitrogen and carbon. If the available N is in excess of that amount necessary to combine with the available carbon for these synthetic purposes, this N appears

in the form of ammonia (as shown in the experiments on tryptophan without glycerol, and in the results obtained by other workers on peptone media containing no carbohydrates); if, however, the amount of available carbon is in excess of the amount necessary to combine with the available N for synthesis, no ammonia appears because all the N is used for synthesis (as shown in our experiments on tryptophan with glycerol, and in the results of other workers on peptone media containing carbohydrates). Hence we believe that carbohydrates far from having a "protein-sparing" effect in the sense used by the above authors, actually enable bacteria to utilise more protein or protein products than they would in the absence of carbohydrates.

II. Experiments on the Decomposition of Tyrosine.

2·5 g. tyrosine were dissolved, by the aid of heat, in 2500 cc. of the basal saline solution described in the tryptophan experiments. After cooling, the medium was distributed as follows:

1. 400 cc. were pipetted accurately into each of two flasks (500 cc. capacity). After sterilisation, these were inoculated with *B. pyocyaneus* and *B. fluorescens* respectively.

2. 400 cc. were pipetted accurately into each of two flasks (500 cc. capacity) and 2 cc. of glycerol were added. After sterilisation these were inoculated with *B. pyocyaneus* and *B. fluorescens* respectively.

3. About 500 cc. were sterilised and used for the estimation of the initial distribution of N in the medium.

4. The residual liquid was placed in a flask, plugged with cotton wool, and autoclaved. Along with the inoculated flasks, this was incubated at 37°, and was used as a standard control solution for the estimation of phenolic groups described later.

Analysis of the Medium.

The method used for determining the *distribution of N* was exactly the same as that described in the tryptophan experiments. However, since tyrosine contains no nitrogen in the phenolic nucleus, another method had to be used for following the fate of this nucleus. This was done by using Folin's colorimetric method for the estimation of phenolic groups [Folin and Denis, 1912, 1915]. The details of our methods of procedure are as follows.

After incubating for a specified number of days, 25 cc. of the inoculated medium were pipetted into a graduated 100 cc. flask (the residue being used for the determination of the distribution of N). To this were added 1 cc. of normal acetic acid and 2 cc. of colloidal iron drop by drop. The flask was heated on a water-bath to flocculate the iron precipitate, cooled, made up to 100 cc., and filtered through a dry filter paper. The phenolic groups present in this solution were then estimated by the use of Folin's phosphotungstic-phosphomolybdic colorimetric reagent, using the control tyrosine solution 4 as a standard phenolic solution.

Table II. *Composition of medium in each case expressed as mg. N per 100 cc.*

Original Medium.

Total N	7·82
Amino-acid N	7·78
Ammonia N	0·06
Phenolic groups equivalent to	100 mg. tyrosine

Tyrosine + Glycerol					Tyrosine without Glycerol				
Incubation period, days			Increase of N and phenolic groups	Decrease of N and phenolic groups	Incubation period, days			Increase of N and phenolic groups	Decrease of N and phenolic groups
				B. pyocyaneus.					
45	Total N ...	7·82	—	—	51	Total N ...	7·82	—	—
	Amino-acid N	0·51	—	7·27		Amino-acid N	0·12	—	7·66
	Ammonia N ...	0·00	—	0·06		Ammonia N ...	4·78	4·72	—
	Synthesised N	3·22	3·22	—		Synthesised N	1·61	1·61	—
	Phenolic groups equivalent to mg. tyrosine...	0·25	—	99·75		Phenolic groups equivalent to mg. tyrosine...	0·14	—	99·86
				B. fluorescens.					
34	Total N ...	7·82	—	—	46	Total N ...	7·82	—	—
	Amino-acid N	0·30	—	7·48		Amino-acid N	0·00	—	7·78
	Ammonia N ...	0·00	—	0·06		Ammonia N ...	3·99	3·93	—
	Synthesised N	4·98	4·98	—		Synthesised N	2·95	2·95	—
	Phenolic groups equivalent to mg. tyrosine...	1·90	—	98·10		Phenolic groups equivalent to mg. tyrosine...	0·00	—	100

Discussion of Results.

From a consideration of these experimental results, the following conclusions may be drawn:

1. As observed in the tryptophan results, relatively large amounts of ammonia but very little synthesised N are formed in the absence of glycerol. In the presence of glycerol, however, the appearance of relatively large amounts of synthesised N is coincident with (in this case) a total absence of free ammonia. This gives further support to our suggested explanation of the so-called "protein-sparing" action of carbohydrates.

2. Both in the presence and absence of glycerol, there is an almost complete disappearance from the tyrosine of phenolic groups giving a colour reaction with Folin's reagent. Since Folin has shown that neither straight chain hydroxy-compounds nor benzene derivatives containing no hydroxyl-group attached to the carbon atoms in the ring give a colour with his reagent, our results may be interpreted in either of the two following ways:

(*a*) That the benzene ring has been ruptured giving rise to straight chain compounds of some sort.

(*b*) That the hydroxyl-group has been removed from the nucleus leaving the benzene ring unbroken.

At present we have no experimental evidence to prove which of these possibilities is correct. It is of interest, however, to note that Traetta-Mosca

[1910] isolated from stable manure a bacillus which in cultural characteristics is closely related to *B. pyocyaneus*. This micro-organism decomposes tyrosine and gives rise, in addition to other compounds, to benzoic acid. Thus in this case there is a removal of the hydroxyl-group from the phenolic nucleus, which would harmonise with our second possibility indicated above.

We are glad to have this opportunity of thanking Professor F. Gowland Hopkins for much kindly criticism and advice during the progress of this work.

REFERENCES.

Folin and Denis (1912). *J. Biol. Chem.* **12**, 239, 245.
—— (1915). *J. Biol. Chem.* **22**, 305.
Kendall and Farmer (1912). *J. Biol. Chem.* **12**, 13, 19, 215, 465, 469.
—— (1913). *J. Biol. Chem.* **13**, 63.
Kendall, Farmer, Bagg, and Day (1912). *J. Biol. Chem.* **12**, 219.
Koser and Rettger (1919). *J. Infect. Dis.* **24**, 301.
Raistrick (1919). *Biochem. J.* **13**, 446.
Traetta-Mosca (1910). *Gazzetta*, **40** (i), 86.

X. DETERMINATION OF THE MINIMUM DOSES OF SOME FRESH CITRUS FRUIT JUICES WHICH WILL PROTECT A GUINEA-PIG FROM SCURVY, TOGETHER WITH SOME OBSERVATIONS ON THE PRESERVATION OF SUCH JUICES.

By ALICE JANE DAVEY.

From the Department of Experimental Pathology, Lister Institute.

(*Received January 5th, 1921.*)

The following account deals with work relating to the preservation of fruit juices over long periods, in such a way as to retain the anti-scorbutic principle as little changed as possible.

The work has been carried out under the direction of Dr Harriette Chick, to whom and to Miss Hume I am indebted for much help and advice during the progress of the experiments.

Many experiments by other workers at the Lister Institute have also been included. Some of them have been published already in a summarised form [Chick, Hume and Skelton, 1918, 1, 2], for others I am indebted to my various colleagues at the Lister Institute.

INTRODUCTION.

Early in 1917, in the course of experiments concerned with the relative anti-scorbutic values of fruit and vegetables, particularly with a view to the needs of the Army, official samples of preserved lime juice, as supplied to the Army and Navy, were tested. The results showed that such preserved lime juices were practically devoid of anti-scorbutic principle, since the largest doses (10 cc. daily), that could be administered, did not indicate the slightest protection of guinea-pigs from scurvy, whereas a very much smaller dose (1·5 cc. daily) of fresh orange or lemon juice will suffice to keep a guinea-pig in good health.

It was thought that the failure of the lime juice might be due to deterioration induced by long keeping or by the method of preservation. Accordingly, tests were instituted on samples of crude juice supplied by the manufacturers and on fresh juice expressed in the laboratory from imported fruit. It was found that the fresh lime juice possessed not more than one quarter of the value of fresh lemon juice [see Chick, Hume and Skelton, 1918, 1, 2].

This result raised the question of the generally received belief in the efficacy of lime juice as a preventive of scurvy and investigations were set on foot to determine the relative anti-scorbutic values of the commoner Citrus fruit juices, and the extent to which these values were diminished by the various methods of preservation used and by long keeping at different temperatures. Attempts have been made to find a method of preserving lemon juice which shall reduce this deterioration to a minimum.

There have already been published the results of the enquiry just referred to, by Chick, Hume and Skelton [1918, 2] into the relative content of anti-scorbutic principle in lemons (*Citrus medica* var. *limonum*) and limes (*C. medica* var. *acida*) where the summarised results include experiments with both guinea-pigs and monkeys. Both sets of experiments clearly demonstrate that "the value of fresh lemon juice is approximately four times that of fresh lime juice" and that preservation and long keeping of lime juice induce considerable loss of its anti-scorbutic principle.

The conflict between experimental results on the one hand and human tradition and practice on the other was resolved by the historical enquiry made by Mrs Henderson Smith [1918, 1919], regarding the use of lime and lemon juice in connection with human scurvy.

More recently, the complete failure of lime juice to afford protection to the army in Mesopotamia is a well-known fact [Willcox, 1920] and later still in Febuary and March 1919, Stevenson [1920] made the same observation of its worthlessness, among the inmates of the Russian civil prisons at Archangel.

PREVIOUS EXPERIMENTAL WORK ON CITRUS FRUIT JUICES.

Old records contain many instances of the great value of oranges and lemons for the prevention and cure of scurvy. One of the most striking is the classical human experiment carried out by Dr Lind [1757; quoted in the Medical Research Committee's Report, 1919, p. 39] by which he showed that oranges and lemons were far superior to other remedies, current in his time.

Some experiments have been carried out by Holst and Frölich [1912] on the anti-scorbutic value of lime and lemon juices, using guinea-pigs as experimental animals. These workers compared freshly squeezed raw lemon juice with commercial samples of lime juice preserved in various ways. The animals were fed on a basal diet of grain and water only; the dose of fruit juice was 5 to 10 cc. daily at most. Comparison was made with other animals receiving no anti-scorbutic. Very little protection was afforded from scurvy and the juice of fresh lemons was found little if at all superior to the preserved lime juices. The addition of the juices to the diet of grain and water prolonged life and mitigated the scurvy symptoms slightly, hence anti-scorbutic in small amount was assumed to be present in the doses given. Holst and Frölich regard this result as lending additional support to the view that guinea-pigs are more susceptible than man to scurvy, and point out that a daily dose of 5 to 10 cc. of lime juice for a guinea-pig, if related to the body weight, is a

proportionally much greater dose than that held to be sufficient to protect a man from scurvy. Since the slight protective effects of the preserved lime juice were not found to be markedly inferior to those of the fresh lemon, the authors inferred that lime juice retains most of its potency after preservation and keeping, and they assume that their experiments justify the widespread belief in the usefulness of preserved lime juice, which they never seem to have called in question.

On the basal diet of grain and water used by Holst and Frölich, with addition of anti-scorbutic in the form of lemon juice, the animals grew very little and hunger symptoms were apparent, since the animals failed to consume the grain. Some of the earliest experiments of Chick and co-workers (Chick and Hume, 1917], showed that if guinea-pigs received a basal diet of oats and bran and water with anti-scorbutic in the form of fruit juice, good health and growth could not be maintained. The animals failed to consume sufficient food and often could not be induced to take their ration of lime or lemon juice without ill effect. The diet cannot be considered equivalent to one in which a ration of fresh cabbage (containing all the accessory food factors) takes the place of the fresh fruit juices. Hence no conclusion drawn from such experiments as to the relative anti-scorbutic potency of, for instance, cabbage and lemon juice can be regarded as valid.

SCOPE OF THE PRESENT PAPER.

The present paper deals first with the determination of the minimum doses of fresh fruit juices, orange, lemon and lime, required to prevent scurvy in guinea-pigs. The results have been used as a basis for finding the loss in anti-scorbutic value which takes place when these juices are preserved or kept for long periods at different temperatures.

Besides an investigation of the keeping properties of carefully preserved laboratory material, the results are also included of tests on several commercial and official samples. Since the true history and mode of preparation of these is not always known the results lack scientific value, but are of very great importance from the practical standpoint.

Some data are also included for the value of chilled fruit. The whole investigation is still not quite complete since few of the specially prepared specimens have been kept as yet for more than two years. Such a period of preservation is however sufficient to give some good indications of the best practical methods to adopt.

TECHNIQUE.

In all particulars the method employed is that described by Chick, Hume and Skelton [1918, 2] and also by Delf and Tozer [1918]. All the experiments have been carried out on young growing guinea-pigs (about 340 g. in weight at the beginning of the experiment) fed on a basal diet of oats and bran, to which was added a ration of 60–90 cc. of cow's milk, autoclaved at 120° for an hour. The fruit juices tested were given as daily doses, administered

by pipette. All food residues were weighed or measured so that the quantities consumed daily could be recorded. The animals were weighed three times weekly. Symptoms of scurvy were noted during life and at post mortem.

The symptoms of guinea-pig scurvy are fully described by Chick, Hume and Skelton [1918, 2] and by Delf and Tozer [1918]; see also the Report of the Medical Research Committee on Accessory Food Factors [1919].

In the present paper the presence or absence of haemorrhages is taken as the decisive symptom in diagnosing scurvy or the reverse. A condition of histological bone lesion, in absence of haemorrhages, is not taken as indicative of scurvy in the light of the results obtained by Tozer [Delf and Tozer, 1918], which show that such a bone lesion frequently also occurs in animals on non-scorbutic diets and is probably due to a deficiency of vitamin *A*.

In order to avoid the ill effects on the animals of large doses of acid juices, all lime juices and certain of the more acid preserved lemon juices were partly, never wholly, neutralised with solid sodium carbonate. Lime juice was rendered more palatable by the addition of cane sugar (1 g. to 10 cc. of juice). Lemon or lime juice doses, whether neutralised or not, were mixed with a little autoclaved milk from the animal's ration before administration. Neutralisation was carried out as short a time as possible before administering doses to the animals, and it is assumed that no appreciable change in the anti-scorbutic value of the ration was so induced [see Harden and Zilva, 1918].

Juices used for experiment were always kept in the Lister Institute refrigerator during experiment in order to keep them as uniform as possible throughout the experiment. The temperature of the refrigerator usually ranged from $-3°$ to $4°$. On rare occasions it went down to $-5°$ and once rose for a week to $18°$.

DETAILS OF EXPERIMENTS AND RESULTS.

I. Determination of Minimum Doses of Fresh Juices.

A. Lemon Juice (*Citrus medica* var. *limonum*).

Sound ripe fruit was selected, any soft or blemished specimens being rejected. The large rough skinned varieties were used and efforts were made to secure uniformity as far as possible. The fruits were halved and the juice expressed by hand on a glass squeezer and strained through coarse muslin or mosquito net with firm squeezing. Juice thus prepared was kept in the refrigerator, but not frozen, during the period of testing. For the earlier experiments [Chick, Hume and Skelton, 1918, 1] supplies were made monthly, so as to be comparable with lime juice from imported fruit, which could not be obtained more frequently. In order to eliminate possible changes due to keeping even for a short time, the juice for the present experiments was prepared fresh every week.

Experiments have been carried out in order to determine within narrow limits, the minimal daily protective dose for a guinea-pig. Early unpublished

experiments, conducted in 1917, show that 10 cc. and 5 cc. daily doses afford ample protection. The paper published in 1918 recorded the effects of 2·5 cc., 1·5 cc. and 0·5 cc. daily doses and 1·5 cc. was found to be the minimal protective dose. The present experiments, using fresher juice, also test the same three doses and reach the same conclusion.

2·5 *cc. daily dose.* Six animals were employed. Two of these died from illnesses other than scurvy after 33 and 37 days respectively. The remaining four animals completed the experimental time and were killed in good health. They were all four in splendid condition, but two of them which had consumed a larger milk ration (85 and 95 cc. respectively, during the last 60 days of experiment), continued to increase in weight to the end of the experimental time and attained a higher maximum weight than did the others.

1·5 *cc. daily dose.* Six animals were used. Of these one died after 30 days of an acute intestinal affection; no scurvy was indicated. The others completed the experimental time (90 days) in good health. The post mortem indicated protection from scurvy.

0·5 *cc. daily dose.* Six animals were used. All developed scurvy. Two of them were killed after 45 days. Attempts were made to cure three of the others with the juice of sweet limes (see p. 90), while the remaining one was treated with canned apricot juice.

The 0·5 cc. daily dose is manifestly insufficient, but some slight protection is indicated because symptoms in life were not diagnosed earlier than the 21st to 28th day, and the length of life in cases where no cure was attempted, was not less than 40 days.

It is concluded therefore that a daily ration of 1·5 cc. fresh lemon juice (never more than one week old) is the minimum daily dose which will protect a guinea-pig from scurvy and this datum is used for the basis for comparison with the anti-scorbutic value of other fruit juices or of different preserved lemon juices. The result of this experiment is very slightly better than that detailed by Chick, Hume and Skelton [1918, 1] where the juice used was kept 0–2 months in the refrigerator, a result which suggests an appreciable though slight loss of anti-scorbutic principle when the juice is kept for such a period even at a low temperature.

B. Lemon Juice with the Rind Oil.

Experiments on preservation which are detailed later had shown that lemon and orange juice, squeezed so as to contain some of the essential oil of the rind, would keep sweet and palatable for long periods, without addition of other preservative, in the same way as lime juice, so treated, does. Lemon and orange juices squeezed without such precaution and without any other preservative, quickly become mouldy and unpalatable. Experiments to determine the minimum dose of such material when fresh are therefore included here with the determination of the minimum doses of the other fresh fruit juices.

The material was prepared in the following way. Whole lemons were sliced by hand and minced in an ordinary kitchen mincer. The juicy pulp thus obtained was squeezed by hand through coarse muslin. The resulting "juice" is a thick creamy emulsion which sets to a soft curd, from which a small amount of clear juice slowly separates. The curd is easily broken up later. This material was bottled without any sterilising precautions and seems to keep well even when the bottles are frequently opened.

As compared with fresh lemon juice expressed without the rind oil, this material is diluted somewhat with the rind constituents, *i.e.* the solid matter of the white inner rind and its watery sap.

The material was kept in the refrigerator (about 0°) during the period of the experiment, *i.e.* about 100 days.

For convenience in measuring small doses of the very thick material, the total quantity required each day was diluted with an equal volume of water; twice the volume of the prescribed dose was then administered to each animal.

1·5 *cc. daily dose.* Four animals were used; one of these showed severe scurvy after 34 days, while another completed 90 days of experiment but showed some indications of scurvy of long standing. Two others were completely protected for 90 days. 1·5 cc. of this juice was not, therefore, quite adequate.

2·5 *cc. daily dose.* Four animals were used of which two died early from intestinal troubles (32 and 34 days). The two remaining animals completed the experiment in good health, without signs of scurvy at post mortem.

The minimum daily dose of this lemon juice with the rind oil (0–10 days old) which will protect a guinea-pig from scurvy is therefore between 1·5 and 2·5 cc.

C. Orange Juice.

The juice was prepared exactly as is described for fresh lemon juice. Some difficulty was experienced in obtaining uniformity owing to the variation in quality of the oranges according to the season of the year. This accounts for certain discrepancies in the results. Oranges deteriorate more rapidly than lemons and late in the season they become dry. The critical experiments on which minimum dose values have been based, were carried out early in the year (Jan. to March), using as far as possible oranges of the same variety (Denia). The juice was prepared once a week, in certain experiments twice a week, and was kept in the refrigerator between successive times of preparation.

Experiments conducted in 1917, using daily rations of 10 cc., 5 cc. and 3 cc., show that these are amply sufficient to protect a guinea-pig from scurvy. Some of these experiments are mentioned in an earlier paper from the Lister Institute [Chick and Hume, 1917]. Further experiments have been conducted and are detailed below, using 1·5 cc. and 0·5 cc. daily doses.

1·5 *cc. daily dose.* Three series of experiments have been conducted at different seasons of the year.

(*a*) Experiment extending from May to July; three animals were used; the juice was expressed weekly. Of the three animals, one died from scurvy in 50 days; another showed "doubtful scurvy" without haemorrhages after 83 days, while the third succumbed to an intestinal illness after 63 days, but was completely protected from scurvy.

(*b*) Experiment extending from March to May; four animals were used; the juice was expressed weekly. Only one animal survived 90 days. It was in excellent health and was completely protected from scurvy. All the other cases were complicated by intestinal infections. No scurvy symptoms were observed during life or at the post mortem, but as the animals died or were killed after 46, 62 and 67 days, the result is not conclusive.

(*c*) Experiment extending from January to March; four animals were used; the juice was expressed twice weekly. One animal died of pneumonia after 67 days but some scurvy was found at post mortem. Two others died from intestinal disorders after 74 and 88 days respectively. At the post mortem one of these showed fragility of bones but no haemorrhages, while the other was without any signs of scurvy. The remaining animal survived 90 days and at the post mortem was found to be normal.

The above results may not appear entirely conclusive; out of a total of eleven animals six were protected from scurvy and of these four survived more than 80 days of experiment. The remaining two, as well as the other partially protected animals, were all victims of infections or illnesses other than scurvy. Experiment (*c*), in which oranges were at their best and the juice used never more than four days old, gives a substantially better result than the others; only in one case was there any scurvy indicated at the post mortem, and that by fragility of the bones only, haemorrhages being absent. In fact three out of the four animals were protected.

0·5 *cc. daily dose.* An experiment with one animal was begun in June 1918. Oranges were then dry and their anti-scorbutic potency lowered as shown by the experiments described above with larger doses. The animal died on the 58th day of experiment with moderately severe scurvy complicated by other illness.

A further experiment was begun in March 1919, the juice being prepared twice weekly. Five animals were employed, all of which showed signs of scurvy round about the 34th day of experiment. One of them showed scurvy with severe haemorrhages and fragile bones after 57 days; another died from disease other than scurvy after 43 days; scurvy haemorrhages were not found but the bones were slightly fragile and the rib junctions nodular. The remaining animals were suffering from severe scurvy from the 57th to the 67th days, as indicated by sore swollen joints, lameness etc. At this time their doses were changed to a daily ration of sweet lime juice which effected a partial cure.

From these experiments it is assumed that 1·5 cc. daily may be considered a minimal protective dose for a guinea-pig, when the freshly expressed juice of oranges at their best, *i.e.* in the early part of the season, is used.

D. Orange Juice with the Rind Oil.

Experiments with lemon juice with the rind oil were so promising that material was prepared in exactly the same way from oranges, in order to establish the minimum dose and test the keeping capacity of this material also.

The fruit was obtained early in March 1919; it was treated exactly as is described for lemons. The emulsion was very thick and set to a substantial curd, a small proportion of juice squeezed without the rind oil was therefore added.

The material was kept in the refrigerator at a temperature about 0° for the time of the experiment, *i.e.* 0–108 days.

1·5 *cc. daily dose.* Four animals were used, of which two were in good health at the end of the experiment. Two died from other causes (63 and 72 days) with very doubtful scurvy, slight bone brittleness but no haemorrhages.

3 *cc. daily dose.* Two animals were used and both were normal at the end of the experiment. The minimum dose of orange juice with the rind oil kept in the refrigerator for 0–108 days was therefore taken to be 1·5 cc. daily.

E. Sweet Lime (*Citrus medica* var. *limetta*).

A small sample of sweet limes was received from Basra in February 1919, sent by Lt.-Col. Ledingham. The sample arrived in poor condition owing to the long time taken in transit. Many of the fruits were useless and the remainder though placed in the refrigerator, deteriorated rapidly and became mouldy and soft. Juice was expressed every two or three days.

It was tested as a cure for animals sick with scurvy, induced by insufficient doses of fresh lemon and orange juices.

Of the six animals used, three had been on a daily ration of 0·5 cc. fresh lemon juice for 49, 52 and 48 days respectively, while three had been on a similar ration of fresh orange juice for 57, 65 and 67 days respectively. All were suffering from severe scurvy as evinced by swollen, painful joints, scurvy position, lameness and falling weight. A daily ration of 2·5 cc. of the juice of sweet limes was substituted for the orange and lemon doses. A very gradual improvement in the condition of the animals became apparent with steadying of the previously falling weight curves; in some cases slight gain in weight took place. Although the cure was very far from complete, the life of the animals was very considerably prolonged. The average life of the six animals in question was 78 days and two of them survived 89 and 91 days respectively; whereas the average length of life of five animals which continued on the small doses of orange and lemon was 49 days only. The post mortem

examination revealed some repair of bone lesions and absence of recent haemorrhages, although signs of severe old haemorrhages were apparent.

Although the sweet limes were old and in very poor condition their juice exercised considerable curative power when administered in 2·5 cc. daily doses to guinea-pigs suffering from severe scurvy. No definite conclusions can be drawn as to the value of sweet limes in good condition, but the result obtained suggests that they should be useful as an anti-scorbutic and superior in this respect to sour limes.

F. Sour or West Indian Lime Juice (*Citrus medica* var. *acida*).

The experiments with fresh ripe lime juice were conducted during the winter of 1917–18 and have been summarised by Chick, Hume and Skelton [1918, 1, 2]. The results are included again here, with some further notes for comparison with the newer results from preserved lime juice and juice from green limes.

The fruit used was obtained through the kindness of Messrs L. Rose & Co. who furnished monthly supplies of specially imported West Indian limes. Owing to war conditions these limes were from one to two months in transit. They do not seem to bear keeping as well as lemons, possibly because of their thinner rind, from which the volatile essential oil is more easily lost.

The juice was prepared exactly as described for lemon and orange juices without the rind oil, only sound firm fruit being employed in its preparation.

The experiment included tests with green unripe limes as well as with ripe limes, as it was thought that the anti-scorbutic value of the green fruit might be greater than that of the ripe fruit. We can draw no very definite line as to what constitutes the limit of ripe and unripe fruit, but ripe fruits were definitely yellow.

Experiments were conducted with 2·5 cc., 5 cc. and 10 cc. daily doses of fresh lime juice. The doses were half neutralised with sodium carbonate, and cane sugar (1 g. per 10 cc. juice) was added to render the juice palatable.

The age of the juice after squeezing varied from 0 to 30 or 40 days old, it being preserved for that time in the refrigerator between squeezings.

2·5 *cc. daily dose.* (*a*) Ripe lime. This amount of ripe lime juice afforded no protection from scurvy. Of seven animals, five died from severe scurvy in 23 to 59 days. The remaining two cases were complicated by other illness and scurvy was less severe after 29 to 67 days, than in the preceding cases.

(*b*) Green lime. When the juice from green limes was employed the results were appreciably better. Scurvy symptoms were milder and the duration of life was longer. One of the four animals used lived for 116 days suffering from slight chronic scurvy, with some improvement towards the end of the experiment, probably correlated with the use of a fresh sample of juice. In another animal which died from infection after 38 days, the post mortem showed no sign of scurvy. The two remaining animals showed scurvy of moderate severity after 56 and 85 days respectively.

5 *cc. daily dose.* (*a*) Ripe lime. Ten animals were used of which three showed definite scurvy and three were normal; two showed doubtful scurvy and two died from other causes (26 and 35 days) without haemorrhages.

(*b*) Green lime. Seven animals were used, of which three died within 34 days from other illness than scurvy and are therefore discounted. As with the ripe lime, practically complete protection was obtained in three of the four cases remaining; the animals survived for over 90 days and no haemorrhages were found at post mortem. The fourth animal showed only doubtful traces of scurvy after 56 days.

10 *cc. daily dose.* Ripe lime only. Doubling of the 5 cc. dose produced no appreciably better result and it failed to eliminate the slight fragility of the bones and enlargement of the rib junctions, with the corresponding histological symptoms, which so frequently occurred but which there is little doubt were due to an insufficiency of vitamin *A*.

Of six animals one was normal in all respects after 88 days of experiment. Another died with severe visceral haemorrhage after 85 days, showing fragility of bones at post mortem. Scurvy was doubtful or absent (no haemorrhages) in two other cases after 52 and 62 days respectively and two died without haemorrhages at 25 and 34 days from other causes.

It must be noted that animals receiving lime juice never grew as vigorously as the animals receiving orange juice or the smaller doses of lemon juice. Healthy animals usually attain a maximum weight of from 550 to 600 g. within the 90 days of experiment, their weight curve approximating nearly to that of animals fed on normal diet. With one exception the highest maximum weight reached by any animal on a 5 cc. or 10 cc. fresh lime juice ration was 470 g. The exception was the single completely protected animal on the 5 cc. daily ration of fresh ripe lime juice, whose weight reached 537 g. on the 78th day of experiment.

The experiments with fresh lime juice indicate 5 cc. daily as the minimal protective dose for a guinea-pig. Taking into consideration the poor growth and consequent low body weight of even those animals which were completely protected from scurvy, it is possible that the 5 cc. daily dose might not ensure protection to better grown animals, whose weight curve more nearly approximated to the normal.

On the other hand, conditions necessitated the use of lime juice prepared monthly from fruits which had suffered from delay in transit. If tests be made from lime juice prepared at more frequent intervals from fresh fruit, the results may indicate a slightly lower value for the minimal dose than has so far been obtained. Pending such further experiments, the minimal protective dose of fresh lime juice required by a guinea-pig cannot be fixed at less than 5 cc. daily, which amount is perhaps best regarded as marginal.

II. Preservation of Fruit Juices.

A. Official and Commercial Samples.

The work carried out with official or commercial samples chiefly concerns the juices of lemon and lime, on account of their use in the rationing of armies, arctic expeditions etc. and the consequent urgent need during the late war for exact information as to their anti-scorbutic value.

Tests have been carried out with a number of official samples as supplied to the Army and Navy, obtained through the authorities. Various commercial samples preserved by different methods have also been tested. Details with regard to a number of such samples have already been published [Chick, Hume and Skelton, 1918, 1]; a few more tests of special samples are added here and the tests of a certain number of similar samples of lemon juice.

Acknowledgments are here made to Messrs L. Rose & Co. and to Messrs Evans Sons, Lescher & Webb for their kindness in supplying samples and furnishing data as to age and method of preparation.

In view of the proved inferiority of lime juice to lemon juice in the fresh state and the uselessness of the preserved lime juices, and having regard also to the promising results obtained with preserved lemon juice, it was recommended to the authorities that steps might be taken to substitute lemon juice for lime juice in supplies to the Army and Navy, thus reverting to the usage of the early part of last century. In response to this suggestion, samples of lemon juices submitted to the authorities have been tested on their behalf.

Since there is some evidence that greater care in the method of preservation will allow retention of more of the anti-scorbutic potency, careful comparison has also been made of the above-mentioned lemon juice with lime juice specially prepared by Messrs Rose, on exactly the same lines as were recommended and adopted in preparing the samples of lemon juice.

A 1. *Official and Commercial Samples of Lime Juice.*

Reference to the table given by Chick, Hume and Skelton [1918, 1, Table I] shows that of six samples of crude lime juice examined, none showed any protection in 5 cc. daily doses, save two samples (Nos. 3 and 4 in table) which did show some small degree of protection. In the case of two of the samples however, even a 10 cc. daily dose conferred no protection.

Two further samples, Nos. 7 and 8, were examined in the course of the present work and yielded a similar result. These were both crude juice supplied by Messrs Rose; the one (No. 7) was prepared from ripe limes in the usual way save that none of the essential oil of the rind was removed before crushing and squeezing; the whole fruit was squeezed right out and the juice, containing more than the usual amount of essential oil, was run straight into the casks for export; two animals on 5 cc. and two on 10 cc. of this material all showed scurvy.

The second sample (No. 8) was ordinary crude lime juice containing no

more than the normal amount of essential oil but prepared from green limes instead of ripe ones. Four animals received 5 cc. of it daily and all showed scurvy. In neither of these cases was the juice more than 2–3 months old when the experiment started.

A third sample (No. 9) was prepared with especial care by Messrs Rose so as to be comparable with the special sample of lemon juice submitted on behalf of the War Office. The material appeared to be crude juice containing the rind oil and 0·07 % SO_2, added as preservative. This sample of lime juice gave the best result ever obtained with lime juice. The lowest value hitherto obtained for the minimum daily dose of lime juice needed to protect a guinea-pig from scurvy is 5 cc., even with juice expressed from imported, though not very fresh limes. With this dose not all guinea-pigs were protected. The special sample, at present under consideration, gave a value rather less than 5 cc., all animals on 5 cc. being perfectly protected; animals on 2·5 cc. all showed scurvy. The inferiority of lime juice is thus still upheld, though in this special case it is considerably less marked than in any other sample ever tested.

A 2. *Official and Commercial Samples of Lemon Juice.*

Four different samples were examined and of these three gave good results, showing a far higher degree of protection on a 5 cc. dose than did the commercial samples of lime juice.

No. 10 was a sample of lemon juice prepared by Messrs Rose for the War Office, squeezed with the rind oil and with $SO_2 = 0{\cdot}1$ % as preservative. This sample corresponds with the lime juice sample No. 9. Protection was obtained in two cases, and a doubtful result in the third case with a 1·5 cc. daily dose of this material, a result practically equal to that obtained with the freshly expressed juice.

No. 11. Lemon juice, expressed without the rind oil, preserved in Messina with 0·25 gal. sulphurous acid to 100 gals. of juice. This juice had been kept for at least six months at room temperature. Only a 5 cc. daily dose was administered and this gave undoubted protection in three cases out of four, the fourth being uncertain. Considerable loss may therefore have taken place but it cannot have been by any means complete.

No. 12. A lemon juice sample furnished by Messrs Evans Sons, Lescher & Webb. It was received in October from lemons squeezed in the preceding spring in Sicily. No preservative was added and apparently none of the rind oil. Only 5 cc. and 10 cc. daily doses were administered and in each case no scurvy developed. Loss, if it had taken place, was therefore not great.

No. 13. A sample of "Kia-Ora" lemon squash, bought in a shop. The history was unknown. Sugar had been added. Doses of 5 and 10 cc. showed no protection from scurvy. This sample therefore ranks with the commercial samples of lime juice, all the other lemon juices being very much superior.

It is quite clear from the results so far given that lemon juice is much

more potent anti-scorbutically than lime juice. It is not however possible to make an accurate quantitative comparison of the keeping capacities of the two, for it is easy to give to guinea pigs a 5 cc. dose of lemon juice, which gives the information whether lemon juice has lost two-thirds of its anti-scorbutic value or not, but it is impracticable to give 15 cc. of lime juice, which is the daily dose necessary in order to obtain the same information about lime juice.

B. Preservation of Special Samples.

The foregoing experiments show clearly (*a*) that orange and lemon juice are more potent as anti-scorbutics than lime juice, and (*b*) that they are on this account more suitable material for use in keeping experiments where gradual loss of anti-scorbutic potency is to be anticipated.

Samples of lemon juice prepared from fresh fruit were stored in the Lister Institute refrigerator, without addition of any preservative. They were tested after an interval of seven months, and it was found that a daily dose of 5 cc. conferred almost complete protection on a guinea-pig. Such juice retained its value even after fermentation of the sugar had taken place, a 5 cc. daily dose again conferring considerable protection. This result stimulated further the enquiry into the methods of preserving and keeping lemon juice, and experiments have been carried out with juice prepared in the laboratory, while others are still in progress. As it was believed that the temperature at which the juice was stored would make considerable difference to the anti-scorbutic, as well as to the gross keeping capacity of the juice, samples were stored at different temperatures. Experiments with samples of lemon juice preserved with varying percentages of sulphite and with the rind oil and of orange juice with the rind oil are also given.

B 1. *Preservation of Lemon Juice by means of "Sulphite"* (potassium metabisulphite).

The juice was expressed from carefully selected fruit, exactly in the manner described for fresh fruit juices. Quantities of from 20 to 30 litres were prepared at one time and the process occupied one or two days. The sulphite was added in 10 % aqueous solution, the juice being stirred vigorously to ensure complete mixing. The juice was bottled immediately, stoppers or corks being sealed with wax so as to be airtight. Two strengths of preservative were tried giving final concentrations of 0·06 % and 0·09 % sulphite in the juice. In the case of a sample of juice to be kept at a higher temperature 0·1 % sulphite was used. It has been found that juice preserved with sulphite does not keep well after bottles have once been opened, hence it is best to store it in bottles containing quantities suitable for a single experiment. Sterilising of bottles previous to filling made no appreciable difference and was accordingly dispensed with.

Samples of this material have been stored for periods of many months, under the following conditions:

(*a*) At ordinary room temperature (12° to 25°), in a dark cupboard in a corridor, not subjected to sunlight.

(*b*) In a hot room at 37°.

(*c*) In the Lister Institute refrigerator, where the temperature was usually slightly above 0° but ranged from − 3° to 4°, once falling as low as − 5° and once (owing to a breakdown) for a week rising to 18°. In winter the juice was occasionally frozen.

The preserved juice is a clear pale yellow liquid containing a small amount of curdy sediment. When efficiently sealed the material does not darken perceptibly after 12 months' keeping in the cold room or at room temperature, but in the hot room considerable darkening takes place in the course of a few weeks although the juice continues for some time to remain good as far as taste and smell are concerned; ultimately however it becomes bad and quite unpalatable. The samples to be tested were removed from the place of storage at the beginning of the experiment and kept in the refrigerator while the experiment progressed.

Tests have been carried out as shown below.

(*a*) Lemon juice preserved with sulphite, kept at room temperature.

1. *Material preserved with* 0·06 % *sulphite.*

Two batches of this material were made. The first batch (No. 14) had been entirely used up after eight months; a second batch (No. 15) was therefore made for further keeping.

Age 3½ *to* 5 *months* (No. 14). After 3½ to 4 months, a 2·5 cc. daily dose failed to protect. After 4 to 5 months a 5 cc. daily dose failed to protect, but the juice appeared to have gone bad, probably due to faulty sealing and all the four animals used developed scurvy as though no anti-scorbutic had been given. On the 19th and 25th days respectively, two of them had their doses replaced by 5 cc. of the juice left over from the last experiment. This seemed to check falling weight in one case but after 14 days both the animals showed advanced scurvy, being thin and miserable. Their dose was then changed to 5 cc. of juice from an unopened bottle of the same material. After 14 days the condition of both animals improved remarkably; there was rise of weight and the use of the hind limbs was regained; they were then killed and showed good condition and repair of bone lesions.

Thus a 5 cc. daily dose, after four months' keeping at room temperature, is in some cases sufficient to effect a cure in cases of advanced scurvy. This indicates a deterioration to about one-third of the original value.

Age 6 *to* 10 *months* (No. 14). A 5 cc. daily dose gave almost complete protection. Of the four animals used, one died from disease other than scurvy in 30 days. Three animals completed the experiment (88–90 days). There

was evidence of slight scurvy, some fragility of bones and slight haemorrhage being found at post mortem.

A further test was carried out using 10 cc. daily. Only one animal was employed. It never made very good growth, remained thin and in poor condition and died after 61 days. Slight fragility of bones was found, but the animal was protected from scurvy.

None of the above-mentioned animals received more than 60 cc. of autoclaved milk daily. Animals receiving large daily rations of the juice preserved with sulphite never made good growth and failed to reach the weights attained by normal animals or by those on small rations of fresh juice. In subsequent experiments the animals received a larger milk ration and the doses of lemon juice were half neutralised with sodium carbonate.

Age 18 *to* 22 *months* (No. 15). Animals receiving a 5 cc. and a 2·5 cc. daily dose showed good health after 60 days; the experiment was unfinished at the time of going to press but it seemed likely that the 2·5 cc. daily dose would afford protection, showing that in this case after nearly two years' keeping at room temperature, lemon juice preserved with 0·06 % sulphite, in some cases at any rate, retains at least three-fifths of its anti-scorbutic potency (the average minimum protective dose of fresh lemon being 1·5 cc.).

2. *Material preserved with* 0·09 % *sulphite.*

Age 7 *to* 12 *months* (No. 16). At seven months a 5 cc. dose effected protection. At 12 months a 2·5 cc. dose failed to do so.

Age 19 *to* 21 *months* (No. 16). At 19 months a 5 cc. dose failed to protect. At 21 months two animals on a 10 cc. dose were in good health after 50 days of experiment; the experiment was still unfinished at the time of going to press.

Material preserved nearly two years with 0·09 % sulphite at room temperature therefore, in this particular set of experiments, showed signs of slow, steady deterioration down to one-fifth or one-sixth of its original value (taking the minimal dose of fresh lemon juice as 1·5 cc.). The various results with lemon juice preserved with sulphite are however rather inconsistent and the different points are more fully discussed later.

(*b*) Lemon juice preserved with sulphite, kept at 37°.

This material was prepared in April 1918 and contained 0·1 % of sulphite. Tests were carried out after three months' keeping, when the juice was already very dark in colour and after 28 months.

Age 3 *months* (No. 17). Four animals received 5 cc. daily. One of these died from infection after 30 days but it is doubtful whether scurvy was beginning. Another animal showed complete protection from scurvy after 67 days. The remaining two survived for 89 days, one with slight scurvy and the other with severe scurvy, the onset of which dated from about the 67th day.

Although insufficient for complete protection the 5 cc. dose was of considerable value.

Age 28 *months* (No. 17). There was no evidence of any protection when a daily dose of 10 cc. was given, the two animals used dying of scurvy in 23 to 30 days. Thus the material when kept at a temperature of 37° showed no anti-scorbutic value after two years; it was also bad in the gross sense.

(*c*) Lemon juice preserved with sulphite, kept in the refrigerator (about 0°).

This material contained 0·06 % of sulphite and was the same as that used as No. 14. Tests were carried out on it after 5 and 27 months' keeping.

Age 5 *months*. A 5 cc. daily dose afforded complete protection to four animals.

Age 27 *months*. A 2·5 cc. daily dose afforded complete protection to two animals. Of two animals receiving 5 cc. one was completely protected and one which became ill from other causes showed slight symptoms of scurvy; doubtless this anomaly, in which the 2·5 cc. dose appears more effective than the 5 cc. was due to the interference of intercurrent disease. In the case of this material stored for more than two years at about 0°, the anti-scorbutic loss seems very slight.

Summary of results, obtained with lemon juice preserved with sulphite.

On reference to the details (Nos. 14, 15, 16) of material stored at room temperature, it is seen that no daily ration of less than 5 cc. was capable of giving adequate protection to a guinea-pig from scurvy, except possibly in the case of the unfinished experiment with 2·5 cc., 22 months old (No. 15). After the juice had been kept only four months, the 5 cc. dose was barely adequate while half this amount was insufficient. Nevertheless, after 18 months' keeping, a 5 cc. daily dose of lemon juice containing 0·06 % of sulphite afforded complete protection.

The effect of the time factor during periods of less than three months has not been investigated. There is considerable loss of anti-scorbutic within this period, since the minimum daily dose of fresh lemon juice is 1·5 cc. daily.

There is no striking difference corresponding with the different percentages of sulphite employed, but the larger amount is probably to be recommended.

The results contain one striking discrepancy, namely that while 5 cc. of lemon juice, containing 0·06 % sulphite, constituted a protective dose after 18 months' keeping, the same dose of similar material, containing 0·09 % sulphite, was powerless to prevent scurvy. Such inconsistencies as this, considered together with the ready tendency of sulphite material to deteriorate after exposure to air, suggest that the sulphite method of preservation is unreliable, in some cases giving good results but in others failing completely.

The keeping experiments at higher temperatures were carried out in order to test the possibility of preserving lemon juice for use in hot climates. As

might be expected, the destruction of anti-scorbutic goes on more rapidly than at lower temperatures. Nevertheless the material was not entirely useless after three months' storage, when a daily dose of 5 cc. afforded considerable protection from scurvy. After 27 months' storage however, no value at all could be detected.

Preservation at lower temperatures increases the time for which the juice will remain efficacious. Extreme limits have not yet been determined. After 27 months' keeping, a 2·5 cc. daily dose of material stored in the cold room protected a guinea-pig from scurvy.

B 2. *Preservation by means of the Rind Oil.*

Experience with lime juice showed that when the juice is expressed from the whole fruit so as to contain the essential oil of the rind it keeps well in the gross sense, without the addition of foreign preservatives. It seemed probable that this method might also serve for other Citrus fruit juices. Small samples of such juice expressed from minced lemons were found to keep well in the laboratory so far as taste and smell are concerned. It remained to test how far the anti-scorbutic value was at the same time preserved.

Accordingly large quantities of material were prepared for testing in the way already described under the determination of the minimum dose for orange and lemon juice with the rind oil. Arrangements were made to carry out tests at considerable periods on material stored at room temperature, at 37° and at a temperature round about 0° (in the Lister Institute refrigerator) in the case of lemon juice, and at 37° and room temperature for orange juice. Material from whatever source was, as usual, stored in the refrigerator during the actual course of each individual test.

(*a*) Lemon juice with the rind oil.

1. *Kept at room temperature.* No. 19.

Material was tested at 4½ months, at 12 to 13 months, and 24 to 27 months.

Age 4½ *months.* A 2·5 cc. daily dose gave full protection showing little or no deterioration to have taken place.

Age 12 *to* 13 *months.* Both 2·5 and 5 cc. daily doses gave protection, showing that after a year at room temperature, little deterioration had still taken place.

Age 24 *to* 27 *months.* The material used in this test had not been kept for the whole time at room temperature, for the first 10 months it was kept at about 0°, later being kept at room temperature. 2·5 cc. failed to give protection. 5 cc. dose gave good health up to 60 days and promised to give full protection though the experiment was incomplete at time of publication.

Even after 10 months at about 0° and 14 to 17 months at room temperature, therefore, the loss in anti-scorbutic value of lemon juice preserved with the

rind oil though perceptible was not very great, and the result compares exceedingly favourably with the lemon juices preserved with sulphite, where only one specimen gave a similar result while other specimens kept for shorter periods failed to protect in larger doses.

2. *Material stored in hot room*, 37°. No. 20.

Under these conditions the juice darkened after a few weeks, but smelt and tasted good after many months. The first samples were not tested till after 24 months' keeping. Two animals received a 2·5 cc. dose, two a 5 cc. dose and one a 10 cc. dose. All showed severe scurvy and there was little if any sign of protection even in the case of the 10 cc. dose. Further experiments with this material were therefore abandoned as it was evident that the method of preservation was useless at such a temperature as 37°.

3. *Material stored in cold room ("chilled"; about* 0°). No. 21.

The material was tested for the first time after 26 months' keeping. Two animals receiving 2·5 cc. daily remained in good health for the full period of experiment. Lemon juice with rind oil preserved at about 0° does not therefore lose a perceptible amount of its anti-scorbutic potency even when so stored for more than two years, a property which it shares with the lemon juice preserved with sulphite when that is stored at the same temperature.

Summary of results with lemon juice preserved with the rind oil.

The juice preserved in this way at 0° and 37° gives much the same result as juice preserved with sulphite. Both show little or no loss of anti-scorbutic potency even after two years at 0° and both scarcely appear to retain any potency after preservation for two years at 37°.

It is in the results at room temperature that the material preserved with rind oil shows its superiority over that preserved with sulphite. In one case, No. 15, the sulphite material appears to be as good, but it evidently cannot be relied upon, particularly after the bottle is opened. There is no evidence that opening the bottle has any destructive effect upon the rind oil material.

(*b*) Orange juice with the rind oil.

The preparation of the material has already been described under the determination of the minimum dose. A portion of the material then tested was set aside for keeping and was preserved at room temperature and at 37°.

Whenever a test was started the portion of material required was kept at about 0° until after the test was finished.

1. *Preserved at room temperature.* No. 22.

Aged 16 *to* 19 *months.* This material was not tested till it was 16 to 19 months old.

Of two animals on a 3 cc. dose one developed scurvy towards the end of the 90 days experiment, the other died from other causes at 63 days without signs of scurvy.

On a 5 cc. dose two animals were in good health after 50 days and one after 22 days, the experiment was unfinished at time of publication but promised well and seemed to suggest that orange juice with the rind oil behaves much in the same way as does lemon juice.

2. *Preserved at* 37°. No. 23.

Aged 16 *to* 17 *months.* Doses of 2·5, 5 and 10 cc. None of these afforded any protection.

Orange juice preserved with the rind oil does not therefore appear to retain its anti-scorbutic potency at 37° any better than does lemon juice preserved in the same way or with sulphite.

III. Preservation of Chilled Fruit, Oranges and Lemons.

At the request of the Food Investigation Board, some tests have been carried out in order to determine the extent to which the anti-scorbutic principle is retained when fruit is preserved by the chilling process, *i.e.* cold storage at temperatures which do not permit of freezing.

Carefully selected samples of oranges and lemons have been kept in cold storage at a temperature of 2·5 to 5·4°. The fruits were wrapped in paper to prevent contact with one another and packed in crates. They were examined periodically and any found to be soft or mouldy were removed. Fluctuations in the temperature of the storage chamber were registered daily by means of a self-recording apparatus. The highest maximum temperature recorded during the period of storage was 5·4° and the lowest minimum was 2·5°.

After periods of several months, a supply of fruit was removed for testing. The juice was prepared as described for fresh fruit every other day, and the fruit and juice were kept in the Lister Institute refrigerator during the period of experiment.

A. *Oranges.* No. 24. The fruit was put into storage in May 1919 and tests were begun in October, 1919, after five months' storage. At the periodic examinations between these dates, 44 % of the original fruit had been rejected. When the testing was begun 96 oranges were available of which only 11 were perfectly sound; all the rest were partly bad but their juice was tested.

Experiments were made with 5 cc., 3 cc. and 1·5 cc. daily doses and were continued for 75 days only. Two guinea-pigs were used for each test. With all three doses complete protection from scurvy was obtained.

After five months' cold storage of the fruit therefore, the anti-scorbutic potency of orange juice is not appreciably impaired, since a daily dose of 1·5 cc. will still protect a guinea-pig from scurvy (minimum dose of fresh orange juice 1·5 cc. daily, see p. 90).

B. *Lemons.* No. 25. The lemons were first tested in January 1920, after nine months' cold storage. During this period they became very mouldy and at different times 108 out of the orignal 238 were discarded. At the time of testing none was completely sound.

Experiments were made with 1·5 cc., 2·5 cc. and 5 cc. daily doses. With the 1·5 cc. daily dose severe scurvy terminated the experiment after 32 days. The 2·5 cc. daily dose gave a similar result. Of two animals receiving 5 cc. daily, one died from lung haemorrhage after 28 days, distinct scurvy being present; the other animal showed sore and painful joints by the 25th day and died from severe scurvy after 51 days.

Thus even the 5 cc. daily dose was practically useless, whereas in the fresh state a 1·5 cc. daily dose of lemon juice affords complete protection from scurvy. The test is not satisfactory because the fruit was not prevented from becoming mouldy. It was in much worse condition and four months older than were the oranges, though neither was in a condition which would ordinarily be regarded as edible. It is probably therefore safe to say that the juice of oranges and lemons, which are preserved by chilling, does not lose appreciably in anti-scorbutic value as long as the fruit is fit for food, but it cannot apparently be kept fit for food in a "chilling" cold store for more than a very few months.

CONCLUSIONS AND SUMMARY.

1. Further experimental evidence is advanced as to the inferiority of lime juice to lemon and orange juice as an anti-scorbutic.

2. The minimum daily doses of the juice of these three Citrus fruits, needed to protect a guinea-pig from scurvy are established as follows:

Lemon	1·5 cc.
Orange	1·5 cc.
Lime	5·0 cc.

These values are used as a basis for comparing the keeping properties, as anti-scorbutics, of the juice of oranges and lemons. The juice is kept for varying times at different temperatures, and in the case of lemon juice preservation with sulphite and with the rind oil is tested, and in the case of orange juice preservation with the rind oil.

3. Preservation with sulphite appears to be satisfactory at a low temperature (about 0°) but at room temperature it seems uncertain and at 37° it is useless, the juice becoming bad in the gross sense as well.

4. Preservation with the rind oil, in case of lemon, is satisfactory and reliable at about 0° and at room temperature. With oranges preservation by this method at 0° was not tried but at room temperature the result was satisfactory. At 37°, both for oranges and lemons, it is as unsatisfactory as is preservation with sulphite, though the juice was not as unpalatable as was the juice preserved with sulphite.

Consequently for 0° and for temperatures about English room temperature, preservation with the rind oil is suggested as the most reliable. There is loss, but even after two years it is not very great.

5. Preservation at 37° has not so far been secured by any method; it is possible that could preservation in the gross sense be secured, the anti-scorbutic property might also be better preserved; it is suggested that this might be attained by boiling the juice first to render it sterile. It has been shown by Delf [1920] that to heat orange juice for an hour at 100° does not materially diminish its anti-scorbutic potency.

6. Experiments are also described on the preservation of oranges and lemons, kept in a chilling (not freezing) cold store; the results are not quite consistent but it appears probable that the anti-scorbutic property is not seriously diminished so long as the fruit is edible.

In conclusion it is my pleasant duty to record my gratitude to Miss Hume, who when I was obliged to resume my university work, undertook the task of final preparation of this paper for the press. Thanks are also due to Miss H. Henderson Smith and to Miss S. Rutherford for assistance in feeding the animals.

A part of the cost of the research was defrayed by the Medical Research Council to whom thanks are also tendered.

REFERENCES.

Chick and Hume (1917). *Trans. Soc. Trop. Med. and Hyg.* **10**, 141.
Chick, Hume and Skelton (1918, 1). *Lancet*, 1918, ii, 735.
—— —— —— (1918, 2). *Biochem. J.* **12**, 131.
Delf and Tozer (1918). *Biochem. J.* **12**, 416.
Delf (1920). *Biochem. J.* **14**, 211.
Harden and Zilva (1918). *Lancet*, 1918, ii, 320.
Henderson Smith (1918). *Lancet*, 1918, ii, 737.
—— (1919). *J. Roy. Army Med. Corps*, **32**, 275.
Holst and Frölich (1912). *Zeitsch. Hyg.* **72**, 1.
Lind (1757). *A Treatise on the Scurvy.* London, 2nd ed. 1757.
Medical Research Council Report (1919). Special Report Series No. 38.
Stevenson (1920). *J. Roy. Army Med. Corps*, **35**, 218.
Willcox (1920). *Brit. Med. J.* 1920, i, 73.

XI. GLYOXALASE IN AVIAN BERIBERI.

By GEORGE MARSHALL FINDLAY.

From the Royal College of Physicians' Laboratory, Edinburgh.

(*Received December 20th, 1920.*)

ALTHOUGH the relationship of beriberi to vitamin B has by now been firmly established there are still those [Walshe, 1918] who maintain that the disease is in reality a toxaemia. This view is by no means new though the original theory has undergone many modifications as our knowledge of beriberi has progressed. At present it is believed that in the absence of vitamin B there is a breakdown in metabolism, more particularly in regard to the metabolism of carbohydrates. Toxins are formed and as a result of their action on the central and peripheral nervous systems, there occur changes very similar to those seen in other forms of toxic polyneuritis. In support of this hypothesis may be cited the curious fact that birds that have been fed on a diet lacking in vitamin B develop symptoms of beriberi more rapidly when their diet contains an excess of carbohydrate. It may, however, be noted that according to Mellanby [1920] carbohydrates have a similar effect in hastening the onset of rickets in puppies deprived of vitamin A. So far, however, no toxin has been isolated from the blood or tissues of animals suffering from beriberi nor has there been demonstrated any interference with the normal metabolism of carbohydrates. In order to investigate the carbohydrate metabolism in avian beriberi the following experiments were carried out on the glyoxalase content of the liver in avian beriberi. Glyoxalase, as was first pointed out by Dakin and Dudley [1913, 1], is an enzyme with an important rôle in the metabolism of sugar. It is widely distributed in nearly all tissues, more especially in the liver and muscles and has the power of transforming "glyoxals" of various compositions into compounds of lactic acid. In the body the chief "glyoxal" so acted upon is pyruvic aldehyde, or as it is sometimes called methylglyoxal. Pyruvic aldehyde is an intermediate stage between glucose and lactic acid. Glyoxalase also plays a part in the conversion of *d*-alanine to lactic acid, while the relationship of pyruvic aldehyde to pyruvic acid is of considerable interest in view of the fact that, as pointed out by Smedley [1912], the higher fatty acids can be synthesised by starting with pyruvic acid. The presence of glyoxalase in the tissues is thus of importance in relation to the metabolism of proteins, fats and carbohydrates.

In the present investigation estimations were made of the glyoxalase content of the livers in control pigeons, in pigeons suffering from beriberi and in pigeons "cured" of beriberi by the administration of vitamin B. Preliminary estimations of the water content of the livers showed that the variation between healthy and beriberic pigeons was practically negligible. The glyoxalase was estimated by the method already employed by Dakin and Dudley [1913, 2, 3], which consists essentially in allowing a 20 % watery extract of the tissues to act on phenylglyoxal, when the phenylglyoxal is converted into mandelic acid according to the equation—

$$C_6H_5.CO.CHO + H_2O = C_6H_5.CH(OH).COOH.$$

The mandelic acid is then extracted and estimated by titration in terms of $N/10$ NaOH.

In the present instance 15 cc. of a 20 % tissue extract were allowed to act on 0·1 g. of phenylglyoxal for 24 hours at 37°. The acidity obtained is shown in the following table:

	Control pigeons	Beriberic pigeons	"Cured" pigeons
Experiment 1	4·2	1·9	2·8
„ 2	4·3	0·9	3·4
„ 3	4·7	2·1	3·0
Average ...	4·4	1·6	3·1

It will thus be seen that in pigeons suffering from beriberi there is as compared with control birds a reduction in the glyoxalase content of the liver by more than one-half. The administration of vitamin B produces a definite rise in the amount of glyoxalase present.

Very similar results were obtained in the case of liver catalase by Dutcher [1918], who believed that vitamin B acted as a co-enzyme for catalase. An attempt was therefore made to determine whether vitamin B acted as a co-enzyme for glyoxalase. For this purpose experiments were performed by adding vitamin B to a watery extract of the liver of beriberic pigeons. The vitamin B was obtained from yeast extract, which had been previously heated to 100° for an hour to destroy its glyoxalase. Three grams of this yeast extract were then added to the mixture of phenylglyoxal and tissue extract and allowed to stand for 24 hours at 37° as before. The average acidity obtained from three observations was 1·72, a result scarcely differing from that found in beriberic birds. Vitamin B can thus only lead to an increased production of glyoxalase when it acts through the intact cell; it cannot therefore be regarded as a true co-enzyme of glyoxalase.

There is thus some evidence to show that in avian beriberi a definite breakdown in carbohydrate metabolism occurs though whether such a breakdown is followed by the production of toxic substances in the tissues is still undetermined.

The writer desires to take this opportunity of thanking Prof. Barger and Mr E. Stedman for their kindness in preparing the phenylglyoxal used in these experiments.

Conclusions.

(1) The glyoxalase content of the liver in pigeons with beriberi is less than that in control pigeons.

(2) The administration of vitamin B to a beriberic pigeon is followed by an increase in the glyoxalase content of the liver.

(3) Vitamin B does not act as a co-enzyme of glyoxalase.

REFERENCES.

Dakin and Dudley (1913, 1). *J. Biol. Chem.* **14**, 155.
—— —— (1913, 2). *J. Biol. Chem.* **14**, 423.
—— —— (1913, 3). *J. Biol. Chem.* **15**, 127
Dutcher (1918). *J. Biol. Chem.* **36**, 63.
Mellanby (1920). *Brit. Med. J.* ii, 328.
Smedley (1912). *J. Physiol.* **45**, 24.
Walshe (1918). *Brit. J. Child. Dis.* **15**, 25

XII. OXIDISING ENZYMES. IV. THE DISTRIBUTION OF OXIDISING ENZYMES AMONG THE HIGHER PLANTS.

By MURIEL WHELDALE ONSLOW.

From the Biochemical Laboratory, Cambridge.

(*Received December 20th, 1920.*)

This and the following paper are a continuation of investigations upon the oxidising enzymes of fruits undertaken for the Food Investigation Board of the Department of Scientific and Industrial Research.

In a previous paper [Onslow, 1920] evidence has been given in favour of the view that many plants contain a representative of a class of enzymes which have been termed oxygenases. It is suggested that these enzymes catalyse the autoxidation of aromatic substances having the dihydroxy grouping of catechol, that is two hydroxyl groups in the ortho-position. Such substances are known to be widely distributed in plants, and certain compounds of this character, such as catechol, caffeic acid, protocatechuic acid, hydrocaffeic acid, etc. have been identified in various genera and species.

The existence of an oxygenase and its aromatic substrate has already been demonstrated in several fruits [Onslow, 1920]. With a view to finding out how general the distribution of these enzymes might be, an examination has been made of plants selected from a number of orders of the Angiosperms. In the case of many orders only one representative has been examined, as the object in view was to ascertain how widely oxygenases are distributed; in certain cases, however, a number of genera in one order have been tested.

Since the reactions of oxidising enzymes may be greatly interfered with by sugars, tannins, organic acids and other substances, the following procedure, already described [Onslow, 1920], has been adopted in making the test. The plant tissue is well pounded in a mortar with 96–98 % alcohol and the mixture filtered on a filter pump. The process is then repeated once or twice with the residue on the filter. In this way the sugars, tannins, etc., are removed, but the enzymes are precipitated and retained in the residue. Previous observations have shown, however, that in some cases the enzyme is not readily extracted from such a residue by water. Hence in testing, the residue itself is divided into two portions in two small evaporating basins. One portion is stirred with dilute catechol solution, the other with water. In

cases where oxygenase is present, browning occurs in the catechol solution and is followed by a blue colour on addition of guaiacum solution. (Before use, the alcoholic solution of guaiacum should be well boiled with a little Merck's charcoal and filtered. This removes any peroxides which may have formed by autoxidation of the guaiacum.)

If no reaction occurs, the control dish is tested with guaiacum and hydrogen peroxide which will determine whether a peroxidase is present or not. If the peroxidase should be absent, which is a rare occurrence, it should be borne in mind that the reaction for oxygenase cannot take place, even though the oxygenase enzyme may be present.

The total number of species examined was 320 and these constitute a selection from the whole of the group of Angiosperms. They represent, moreover, 180 Natural Orders and 309 genera. Of the above species, 16 (*i.e.* 5 %) representing 16 genera and 13 Natural Orders, failed to give a peroxidase reaction with hydrogen peroxide and guaiacum. In order to ascertain satisfactorily whether they contain oxygenase by the catechol-guaiacum test, it would be necessary in these cases to add peroxidase from another source. This has not been tried, so that in estimating the percentage of "positive orders" (*i.e.* those containing oxygenase) these 16 species must be eliminated. Some of the latter occur in Natural Orders (marked * in the following list) of which other representatives have been examined, so that in fact only 7 orders remain to be cancelled on the above grounds. They are Bruniaceae, Ebenaceae, Empetraceae, Frankeniaceae, Myrsinaceae, Pyrolaceae and Sarraceniaceae. It should strictly be understood that further observations on these species, or on additional genera and species, may show that these orders might be ranked as positive and they are merely eliminated from the present set of observations.

Taking these revised figures of 304 species, 173 Natural Orders and 293 genera, the percentages of positive orders are distributed in the various sub-groups as follows:

	Percentage of orders examined containing oxygenase
Angiosperms:	
Of the total number of orders (286), 60 % were examined	62
Monocotyledons:	
64 % examined	76
Dicotyledons:	
60 % examined	60
Archichlamydeae:	
57 % examined	51
Sympetalae:	
71 % examined	84

The following is a list of orders examined. Those in italics contain species with oxygenase, *i.e.* are positive orders. The numbers in brackets denote the species examined; the second numbers the species with oxygenase.

Monocotyledoneae.

Pandanales. Typhaceae (1), 0; *Pandanaceae* (2), 2; *Sparganiaceae* (1), 1.
Helobiae. *Potamogetonaceae* (1), 1; Aponogetonaceae (1), 0; Scheuchzeriaceae (1), 0; *Alismaceae* (1), 1; *Butomaceae* (1), 1; *Hydrocharitaceae* (1), 1.
Glumiflorae. *Gramineae* (1), 1; *Cyperaceae* (1), 1.
Principes. *Palmae* (1), 1.
Synanthae. *Cyclanthaceae* (1), 1.
Spathiflorae. *Araceae* (1), 1; *Lemnaceae* (1), 1.
Farinosae. *Bromeliaceae* (2), 1; *Commelinaceae* (1), 1; Pontederiaceae (1), 0.
Liliflorae. *Juncaceae* (1), 1; *Stemonaceae* (1), 1; Liliaceae (3), 0; Amaryllidaceae (2), 0; *Taccaceae* (1), 1; *Dioscoreaceae* (1), 1; Iridaceae (1), 0.
Scitamineae. *Musaceae* (1), 1; *Cannaceae* (1), 1; *Marantaceae* (1), 1.
Microspermae. *Orchidaceae* (1), 1.

Dicotyledoneae (Archichlamydeae).

Verticillatae. *Casuarinaceae* (1), 1.
Piperales. *Piperaceae* (1), 1; *Chloranthaceae* (1), 1.
Salicales. *Salicaceae* (2), 2.
Garryales. *Garryaceae* (1), 1.
Juglandales. *Juglandaceae* (1), 1.
Fagales. Betulaceae (1), 0; Fagaceae (2), 0.
Urticales. *Ulmaceae* (1), 1; *Moraceae* (2), 2; *Urticaceae* (1), 1.
Proteales. *Proteaceae* (1), 1.
Santalales. *Santalaceae* (1), 1; Olacaceae (1), 0; *Loranthaceae* (1), 1.
Aristolochiales. *Aristolochiaceae* (1), 1.
Polygonales. Polygonaceae (1), 0.
Centrospermae. *Chenopodiaceae* (3), 2; Amarantaceae (1), 0; *Nyctaginaceae* (1), 1; Phytolaccaceae (1), 0; *Aizoaceae* (1), 1; *Basellaceae* (1), 1; *Caryophyllaceae* (4), 1.
Ranales. Nymphaeaceae (1), 0; *Ranunculaceae* (11), 9; *Lardizabalaceae* (1), 1; *Berberidaceae** (1), 1; *Menispermaceae* (1), 1; *Magnoliaceae* (1), 1; *Calycanthaceae* (1), 1; *Anonaceae* (1), 1; *Lauraceae* (1), 1.
Rhoeadales. *Papaveraceae* (4), 4; Capparidaceae (1), 0; Cruciferae (8), 0; Resedaceae (1), 0; *Moringaceae* (1), 1.
Sarraceniales. Nepenthaceae (1), 0.
Rosales. Crassulaceae (1), 0; *Saxifragaceae** (3), 1; *Pittosporaceae* (1), 1; Hamamelidaceae (1), 0; Eucommiaceae (1), 0; *Platanaceae* (1), 1; *Rosaceae* (17), 17; Leguminosae (5), 0.
Geraniales. Geraniaceae* (1), 0; Oxalidaceae (1), 0; Tropaeolaceae (1), 0; *Linaceae* (1), 1; *Erythroxylaceae* (1), 1; Zygophyllaceae (1), 0; Rutaceae (2), 0; Simarubaceae (1), 0; Meliaceae (1), 0; Malpighiaceae (1), 0; Polygalaceae (1), 0; Euphorbiaceae (2), 0; *Callitrichaceae* (1), 1.
Sapindales. Buxaceae (1), 0; Coriariaceae (1), 0; *Anacardiaceae* (1), 1; Corynocarpaceae (1), 0; *Aquifoliaceae* (1), 1; Celastraceae (1), 0; Staphyleaceae (1), 0; Aceraceae (1), 0; *Hippocastanaceae* (1), 1; Sapindaceae (1), 0; Melianthaceae (1), 0; Balsaminaceae (1), 0.
Rhamnales. Rhamnaceae (1), 0; *Vitaceae* (2), 2.
Malvales. Elaeocarpaceae (1), 0; Tiliaceae (1), 0; Malvaceae (2), 0; *Sterculiaceae* (2), 2.
Parietales. Dilleniaceae (1), 0; *Eucryphiaceae* (1), 1; *Ochnaceae* (1), 1; *Marcgraviaceae* (1), 1; *Theaceae* (1), 1; *Guttiferae* (1), 1; Tamaricaceae (1), 0; Cistaceae (1), 0; Violaceae (1), 0; *Flacourtiaceae* (1), 1; *Stachyuraceae* (1), 1; Passifloraceae (1), 0; Caricaceae (1), 0; *Loasaceae* (1), 1; Datiscaceae (1), 0; Begoniaceae (1), 0.
Opuntiales. *Cactaceae* (3), 3.
Myrtiflorae. Thymelaeaceae (1), 0; *Elaeagnaceae* (1), 1; Lythraceae (1), 0; Punicaceae (1), 0; *Alangiaceae* (1), 1; Combretaceae (1), 0; Myrtaceae (1), 0; Onagraceae (2), 0; *Hippuridaceae* (1), 1.
Umbelliflorae. *Araliaceae* (2), 2; *Umbelliferae* (5), 5; *Cornaceae* (2), 1.

DICOTYLEDONEAE (Sympetalae).

ERICALES. *Clethraceae* (1), 1; *Ericaceae** (4), 3; Diapensiaceae (1), 0.
PRIMULALES. Theophrastaceae (2), 0; Primulaceae* (3), 0.
PLUMBAGINALES. Plumbaginaceae (1), 0.
EBENALES. Sapotaceae* (1), 0; *Styracaceae* (1), 1.
CONTORTAE. *Oleaceae* (3), 3; *Loganiaceae* (1), 1; *Gentianaceae* (2), 2; *Apocynaceae* (3), 2; *Asclepiadaceae* (1), 1.
TUBIFLORAE. *Convolvulaceae* (1), 1; *Polemoniaceae* (3), 2; *Hydrophyllaceae* (1), 1; *Boraginaceae* (4), 4; *Verbenaceae* (2), 2; *Labiatae* (3), 3; *Nolanaceae* (1), 1; *Solanaceae* (7), 7; *Scrophulariaceae* (14), 14; *Bignoniaceae* (1), 1; *Martyniaceae* (1), 1; *Orobanchaceae* (1), 1; *Gesneriaceae* (6), 6; *Globulariaceae* (1), 1; *Acanthaceae* (1), 1; *Myoporaceae* (1), 1.
PLANTAGINALES. *Plantaginaceae* (1), 1.
RUBIALES. *Rubiaceae* (2), 2; *Caprifoliaceae* (5), 5; *Valerianaceae* (1), 1; *Dipsacaceae* (2), 2.
CUCURBITALES. *Cucurbitaceae* (2), 1.
CAMPANULATAE. Campanulaceae (2), 0; *Compositae* (13), 13.

The observations recorded give an idea of the extent to which the enzymes of the oxygenase type are distributed, and, on the whole, there appears to be a connection between systematic relationship and the presence of oxygenase. Either the presence, or absence, of these enzymes is frequently characteristic of an order. A certain percentage of orders, on the other hand, has been found to contain both positive and negative genera, and this percentage would probably increase if more genera were examined. No definite case of positive and negative species within a genus has been noted, but the data on this point are very limited.

The conclusions which may be drawn are as follows. Enzymes of the oxygenase type are present in the aerial parts of about 62 % of the orders of Flowering Plants examined[1]. Considering the groups in detail, they are widely distributed in the Monocotyledons (76 % of the orders examined); in the Dicotyledons, they are less frequent in the Archichlamydeae (51 %) than in the Sympetalae (84 %). In most of the great cohorts of the Archichlamydeae there is a mixture of positive and negative orders in practically equal numbers, but in the Sympetalae nearly all the orders are positive. It has been suggested, on botanical evidence, that the main lines of evolution among the Sympetalae had their origin among the Archichlamydeae. It would appear that the presence of an oxygenase system is characteristic of plants of the dominating lines of evolution among the Dicotyledons, as practically all the most highly developed orders are positive. Hence these lines may have taken origin from positive stock among the Archichlamydeae.

Though on the whole there is a distinct correlation between presence of oxygenases and systematic relationship, it appears possible that in any genus or species there may be lack of an oxygenase due to some form of metabolism peculiar to that individual. This may not indicate a different origin phylogenetically, as for instance the case of the Campanulaceae, which, though

[1] Many of the remaining orders uninvestigated are rare and it is difficult to obtain representative genera; or they may contain only a few genera. All the larger and widely represented orders have been considered.

negative, are doubtless related to phyla and groups which are characteristically positive.

The extent of the distribution of the catechol substances which act as substrates for the oxygenases has not been so widely investigated. About 30 species, mostly of different genera, which give the oxygenase reaction have been found to contain substances giving the catechol reaction. About 15 species, also chiefly representatives of different genera, which give no oxygenase reaction were found to be devoid of catechol compounds. No case has been noted yet in which oxygenase is present and no catechol compound, though the converse, absence of oxygenase and presence of catechol compound, appears to be true of one or two plants examined.

In the 38 % of negative orders, presumably the oxygenase-catechol system is absent. With respect to this the following points may be considered. It has already been insisted upon repeatedly that water extracts of tissues are usually quite unreliable as material for testing. The same may be true of the actual tissue itself, owing to inhibiting substances present. Even after treatment with alcohol, the readiness with which the tissue residue gives the catechol-guaiacum reaction varies within very wide limits. Hence it is conceivable that in negative species there may still be some inhibitor in the tissue residue, or some inhibiting condition in the experiment, and that the presence of the system cannot be detected by the enzyme method. The enzyme, catalase, might represent such an inhibitor; though almost universally distributed, it might under certain conditions decompose all available peroxide. Experiments to detect such inhibitors have not been successful. Additional determinations of the presence or absence of catechol compounds in negative cases would decide whether the system is absent altogether or not.

Browning and discoloration of the tissues, occurring after death brought about by mechanical injury, chloroform vapour, etc., which was originally thought to accompany the presence of the oxygenase-catechol system, has been found not to be an infallible guide, as it may be inhibited in the same way as the guaiacum reaction. It may also occur in plants in which no oxygenase could be detected (*Vicia*, *Aucuba*) and this point will be referred to again.

It should be borne in mind that the discoloration of tissues, when it does occur, is probably not only due to the oxidation of the catechol compounds but may be augmented by the oxidation of any other aromatic substances present in the tissue. For the system, peroxide-peroxidase, when once established, can oxidise compounds of the most varied types.

Examinations, on a considerable scale, of the guaiacum reaction with plant juices have been conducted by Passerini [1899] and also by Clark [1911]. The former observer examined representatives of about 49, the latter of about 72 orders. Their results agree on the whole with those described here, though fewer direct reactions were obtained on account of fresh juices and water extracts having been employed by them.

In the examination described in the present paper, leaves and leafy shoots were nearly always used as material. Usually the enzyme and substrate are more or less distributed throughout the plant. In some cases as in the raspberry (*Rubus Idaeus*), the blackberry (*R. fruticosus*) and the tomato (*Solanum Lycopersicum*) it was noted that the fruit is negative though the plant is positive. In one case, the fig (*Ficus Carica*), it was noted that the fruit was positive and the leaves negative.

No roots have been examined. Passerini examined various roots and with respect to these organs positive results are recorded by him for species described as negative in the present paper. Possibly root examination might throw further light on distribution.

As far as evidence goes, the conclusion may be drawn that catechol oxygenases, and most probably catechol compounds also, are widely distributed among the Flowering Plants. Their distribution is not apparently correlated with any particular habit or morphological characteristic, as it occurs alike in water and land plants, in trees, shrubs and herbs, in annuals and perennials, in parasites, etc. The only outstanding fact is the almost universal presence of oxygenase in the most highly developed orders, of which the herbaceous, in contrast to the woody, habit is also a characteristic. Hence there is the possibility that there may be a connection between the herbaceous habit and the distribution of the catechol-oxygenases.

The physiological significance of the catechol-oxygenase system is obscure. It is no doubt of the nature of those described by Palladin and others as being involved in respiration processes; such systems, by means of converting molecular into active oxygen, may oxidise substances produced in respiration. If so, one would expect to find the oxygenases universally distributed. No evidence has yet been obtained of an extensive distribution of any other similar system. The bean (*Vicia Faba*) and the Aucuba Laurel (*Aucuba japonica*), however, blacken after death and apparently do not contain a catechol oxygenase. Hence they and other plants may contain a different type of oxidising enzyme.

SUMMARY.

(1) Representatives of a number of orders of the Angiosperms (about 60 % of the total) were examined for an oxygenase which would catalyse the oxidation of catechol. About 62 % of the orders examined were found to contain species with such an enzyme.

(2) With regard to sub-groups of the Angiosperms, such oxygenases are widely distributed in the Monocotyledons, being present in 76 % of the orders examined. In the Dicotyledons such enzymes are less frequent in the Archichlamydeae (51 % of the orders examined) than in the Sympetalae (84 % of the orders examined).

REFERENCES.

Clark (1911). *Torreya*, **11**, 23, 55, 84, 101.
Onslow (1920). *Biochem. J.* **14**, 535.
Passerini (1899). *Nuov. Giorn. Bot. Ital.* **6**, 296.

XIII. OXIDISING ENZYMES. V. FURTHER OBSERVATIONS ON THE OXIDISING ENZYMES OF FRUITS.

By MURIEL WHELDALE ONSLOW.

From the Biochemical Laboratory, Cambridge.

(*Received December 20th, 1920.*)

The following is an account of the examination for oxidising enzymes of a further selection of fruits.

The results are in general accordance with those already obtained [Onslow, 1920]. Of the fruits examined, the following were found to contain the components of an oxidase: cherry, peach, apricot, medlar, strawberry, grape, fig and mulberry. In others, the blackberry, red currant, black currant, gooseberry, grape fruit, pomegranate, pineapple, melon and tomato, only a peroxidase could be detected. These results are not contrary to what may be expected on the basis of natural classification; they also exemplify a point mentioned in the former paper, that the enzyme content of the fruit is not always the same as that of the plant itself. Blackberry and tomato leaves, for instance, contain an oxidase, whereas the ripe fruit does not.

The experimental methods employed were those described in the author's previous paper [Onslow, 1920]. Pieces of fresh tissue of the fruits were placed in guaiacum tincture, both with and without hydrogen peroxide; also in solutions of benzidine and of α-naphthol, both with hydrogen peroxide. Water extracts of the tissues (after neutralisation, if acid) were also tested with the same reagents. To remove inhibitors, fresh tissue was pounded thoroughly with 96–98 % alcohol, sucked dry on a filter funnel, and the process repeated. The residue ("tissue residue") was extracted with water; the filtrate ("enzyme extract") was tested for peroxidase with guaiacum and hydrogen peroxide (and if this test were negative, with benzidine and hydrogen peroxide), and for oxygenase (peroxidase being present) with catechol solution and guaiacum. If the extract failed to give any reaction, the tissue residue itself was suspended in the reagents. Finally, a hot alcoholic extract of the fruit was made, filtered, and the alcohol distilled off *in vacuo*. The residue was precipitated with solid lead acetate, and the precipitate formed filtered off. The latter was decomposed with the minimum amount of dilute sulphuric acid, filtered from lead sulphate and neutralised. This extract ("aromatic extract") was tested for the catechol reaction, and, if positive, was treated with enzyme extract (or tissue residue if the extract gave no result) and guaiacum. If organic acids and tannins were present, the former were precipitated with calcium chloride and alcohol, the latter with gelatin.

Some points which have already been mentioned in previous papers may be again emphasised. First, discoloration of tissues cannot be relied upon in every case for an indication of the presence of the system. The simultaneous presence of a certain amount of various substances, such as tannins, organic acids, sugars, etc., may inhibit the discoloration, though the components for its production are present. This is true, for instance, of the apricot, of which the flesh does not discolour on injury, though the enzyme system present is similar to that in the peach, plum, cherry and many others, all of which do discolour on injury.

A second point to be emphasised is that inhibitors may, in the same way, render water extracts of tissues, or even the tissue itself, quite unreliable material with which to test for enzymes. The tissues should be extracted with alcohol which removes the inhibiting substances and precipitates the enzymes in the tissue residue.

A third point is that in some fruits it is difficult or impossible to extract the enzymes from the tissue residue; hence the action of the peroxidase on guaiacum, and of the oxygenase on catechol or the plant aromatic substance, can only be demonstrated by placing the tissue residue itself in the solution. Fruits which show this phenomenon contain tannin, and it is suggested that the tannin may cause precipitation and adsorption of the enzymes, thereby rendering them less soluble. This phenomenon is shown in various degrees by the medlar, quince, cherry and apple.

A similar phenomenon was also observed in another type of fruit, namely that in which the tissues are much disintegrated on ripening, and in which there is frequently much pectin substance. Such are the strawberry, gooseberry, red and black currants and, to some extent, the grape. From the tissue residue consisting chiefly of pectinogen, either no or very little extraction of enzymes could be obtained with water in the case of the gooseberry and currant; neither peroxidase nor oxygenase could be extracted from the strawberry and only peroxidase from the grape.

Employing the above methods, the following results have been obtained:

X. The cherry, *Prunus Cerasus* (Rosaceae), contains an oxidase (oxygenase, catechol substance and peroxidase).

A slightly pigmented variety was used. Discolours on injury. *Tissues and their water extract:* guaiacum alone and benzidine (H_2O_2), positive; α-naphthol (H_2O_2), negative. *Water extract of tissue residue:* peroxidase and oxygenase reactions, positive. *Aromatic extract:* catechol reaction, positive; added to enzyme extract (or tissue residue) and guaiacum, negative. After precipitation with calcium chloride and alcohol, again added to tissue residue and guaiacum, positive. Tannin present.

XI. The peach, *Prunus Persica* (Rosaceae), contains an oxidase.

Discolours on injury. *Tissues and their water extract:* guaiacum alone and other two tests, positive. *Water extract of tissue residue:* peroxidase and oxygenase reactions, positive. *Aromatic extract:* catechol reaction, positive; added to enzyme extract (or tissue residue) and guaiacum, negative. After precipitation with calcium chloride and alcohol, again added to enzyme extract and guaiacum, positive.

XII. The apricot, *Prunus Armeniaca* (Rosaceae), contains an oxidase.

Does not discolour on injury. *Tissues and their water extract:* guaiacum alone and other two tests, positive. *Water extract of tissue residue:* peroxidase and oxygenase reactions, positive. *Aromatic extract:* catechol reaction, positive; added to enzyme extract and guaiacum, positive.

XIII. The medlar, *Mespilus germanica* (Rosaceae), contains an oxidase. The oxygenase cannot readily be extracted by water from the tissue residue, but reacts as if adsorbed to this residue. Sufficient peroxide is formed in a water extract of the crushed tissues to give a reaction with benzidine alone[1].

Fruit examined in October before it becomes soft. Discolours rapidly on injury. *Tissues and their water extract:* guaiacum alone and other two tests, positive. Water extract, positive reaction with benzidine alone. *Water extract of tissue residue:* peroxidase reaction, positive; oxygenase reaction, negative. *Tissue residue:* oxygenase reaction, positive. *Aromatic extract:* catechol reaction, positive; added to tissue residue and guaiacum, negative. Tannin present. Precipitation with calcium chloride and alcohol not tried.

XIV. The strawberry, *Fragaria elatior* (Rosaceae), contains an oxidase, though the reactions are not very readily obtained. Neither the oxygenase nor the peroxidase can be extracted by water from the tissue residue, but both act as if adsorbed to the residue.

Does not discolour on injury. *Tissues:* all three tests negative. *Water extract* (after neutralisation): all three tests negative. *Water extract of tissue residue.* If fruits are smashed and squeezed through muslin to remove seeds, and juice and pulp stirred up with excess of alcohol, a bulky gelatinous precipitate of pectinogen mixed with tissue residue is obtained. Treated again with alcohol. Residue is largely pectinogen and swells up in water, but water extract is negative for peroxidase and oxygenase reactions. *Tissue residue:* peroxidase and oxygenase reactions, positive though not given very readily. *Aromatic extract:* catechol reaction, positive though abnormal; added to tissue residue and guaiacum, negative. After treatment with calcium chloride and alcohol, still negative.

XV. The blackberry, *Rubus fruticosus* (Rosaceae), contains a peroxidase.

All colour reactions difficult on account of anthocyan pigment. *Water extract of tissue residue:* all three tests negative. *Tissue residue:* only benzidine (H_2O_2) positive. *Aromatic extract:* catechol reaction, doubtful owing to the presence of an iron-blueing substance, probably a tannin.

XVI. The red currant, *Ribes rubrum* (Saxifragaceae), contains a peroxidase. This cannot be extracted by water from the tissue residue but behaves as if adsorbed to the residue.

Does not discolour on injury. *Tissues:* guaiacum (H_2O_2), negative; other two tests positive. *Water extract* (after neutralisation): only benzidine (H_2O_2) positive. *Water extract of tissue residue:* peroxidase and oxygenase reactions, negative. *Tissue residue:* benzidine (H_2O_2) and α-naphthol (H_2O_2), positive. *Aromatic extract:* catechol reaction, negative.

XVII. The black currant, *Ribes nigrum* (Saxifragaceae), contains a peroxidase. This cannot be extracted by water from the tissue residue but behaves as if adsorbed to the residue.

Colour reactions difficult on account of anthocyan pigment. *Water extract of tissue residue:* peroxidase and oxygenase reactions, negative. *Tissue residue:* only benzidine (H_2O_2) positive.

[1] The experiment of adding a solution of catechol oxidised by an oxygenase, together with a peroxidase, to benzidine has also been tried with a positive result.

XVIII. The gooseberry, *Ribes Grossularia* (Saxifragaceae), contains a peroxidase. As in the case of the currant, it cannot be extracted from the tissue residue.

Both red and green varieties used, and reactions may vary with the variety employed. Does not discolour on injury. *Tissues:* guaiacum (H_2O_2), negative; other two tests, positive. *Water extract of tissues* (from green variety after neutralisation): two latter tests positive. *Water extract of tissue residue:* peroxidase and oxygenase reactions, negative. *Tissue residue* (tested only in red variety): all peroxidase tests positive. *Aromatic extract:* catechol reaction, negative.

XIX. The grape fruit, *Citrus decumana* (Rutaceae), contains a peroxidase.

Tissues: (rind), all peroxidase tests positive; (flesh), two latter only positive. *Water extract of tissues* (after neutralisation): almost completely negative results. *Water extract of tissue residue:* only benzidine (H_2O_2) positive. *Tissue residue:* all peroxidase tests positive. *Aromatic extract:* catechol reaction, negative.

XX. The pomegranate, *Punica Granatum* (Punicaceae). The inner tissues contain a peroxidase. A substance giving the catechol reaction is present and is probably a tannin.

Tissues (fleshy part of seed): guaiacum (H_2O_2), negative; other two tests positive. *Water extract* (after neutralisation): only benzidine (H_2O_2) positive. *Water extract of tissue residue:* peroxidase and oxygenase reactions, negative. *Aromatic extract:* catechol reaction, positive though abnormal: tannin present.

XXI. The grape, *Vitis vinifera* (Vitaceae), contains an oxidase. Neither the oxygenase nor peroxidase is readily extracted by water from the tissue residue but both act as if adsorbed to this residue.

Both purple and white varieties were used. Results seem to vary in detail with the variety used. Discolour on injury. *Tissues:* guaiacum alone and other two tests, positive. *Water extract* (after neutralisation): only benzidine (H_2O_2) positive (white variety). *Water extract of tissue residue:* only benzidine (H_2O_2) positive; oxygenase reaction, positive in white variety only. *Tissue residue:* peroxidase and oxygenase reactions, positive. *Aromatic extract:* catechol reaction, results on the whole positive, especially in the white variety: added to enzyme extract (or tissue residue) and guaiacum, negative; also after precipitation with calcium chloride and alcohol.

XXII. The pineapple, *Ananas sativus* (Bromeliaceae), contains a peroxidase.

Does not discolour on injury. *Tissues and their water extract:* positive with all three peroxidase tests. *Water extract of tissue residue:* peroxidase reaction, positive; oxygenase reaction, negative. *Aromatic extract:* catechol reaction, negative.

XXIII. The melon, *Cucumis Melo* (Cucurbitaceae), contains a peroxidase.

Does not discolour on injury. *Tissues and water extract:* positive with all three peroxidase tests. *Water extract of tissue residue:* peroxidase reaction, positive; oxygenase reaction, negative. *Aromatic extract:* catechol reaction, negative.

XXIV. The tomato, *Solanum Lycopersicum* (Solanaceae), contains a peroxidase.

Does not discolour on injury. *Tissues and water extract:* positive with all three peroxidase tests. *Water extract of tissues:* peroxidase reaction, positive; oxygenase reaction, negative. *Aromatic extract:* catechol reaction, negative.

XXV. The fig, *Ficus Carica* (Moraceae), contains an oxidase.

Discolours on injury. *Tissues and water extract:* guaiacum alone and other two tests, positive. *Water extract of tissue residue:* peroxidase and oxygenase reactions, positive. *Aromatic extract:* catechol reaction, positive.

XXVI. The mulberry, *Morus nigra* (Moraceae), contains an oxidase.

Discolours somewhat on injury. *Tissues:* guaiacum alone and other two tests, positive. *Water extract* (after neutralisation): no satisfactory reaction, and observation difficult because of anthocyan pigment. *Tissue residue:* peroxidase and oxygenase reactions, positive. *Aromatic extract:* catechol reaction, difficult to detect on account of anthocyan pigment; added to enzyme extract (or tissue residue) and guaiacum, negative. After precipitation with calcium chloride and alcohol, again added to tissue residue and guaiacum, positive.

I take this opportunity of recording my thanks to Dr F. F. Blackman, F.R.S., for help and advice in connection with this and the preceding paper.

REFERENCE.

Onslow (1920). *Biochem. J.* 14, 541.

XIV. THE EXTRACTION OF MELANIN FROM SKIN WITH DILUTE ALKALI.

By WILLIAM JOHN YOUNG.

The Australian Institute of Tropical Medicine, Townsville, N. Queensland.

(*Received December 23rd, 1920.*)

It has generally been assumed that the black pigments (melanins) which occur normally or pathologically in animal tissues, hair, skin, feathers, etc. are chemically very inert substances, and in most cases their isolation has been carried out by dissolving the tissue in boiling alkalies or strong acids. Thus Abel and Davis [1896] prepared melanins from both the skin and hair of a negro by heating the material at 100° either with 5–6 % potassium hydroxide or with concentrated hydrochloric acid until all the keratin had dissolved. Other investigators have used even more drastic methods such as boiling with more concentrated alkalies or even fuming hydrochloric acid.

That some melanins are really not so resistant to alkalies and acids was shown by Gortner [1910] who investigated the effects of alkalies upon the melanin of black-sheep's wool. He extracted the pigment from the wool by boiling with successive portions of a 0·2 % solution of sodium hydroxide, and from the first two or three extracts he obtained a melanin of constant composition, C = 52·57 %, H = 7·28 %, N = 13·43 %, S = 1·33 %. This substance when purified contained no ash, was soluble in alkali and in very dilute mineral acids (*N*/20), and was precipitated by stronger acids. After boiling this with stronger alkali a pigment could be precipitated by acid from the solution, which contained less nitrogen and hydrogen than the original, and was no longer soluble in dilute acids.

This acid-soluble pigment was only obtained from the first two or three extractions, the subsequent extracts yielding a different pigment, insoluble in acid and containing less nitrogen and hydrogen, and Gortner considered this to be a decomposition product of the acid-soluble pigment.

When stronger solutions of alkali were employed for the extraction, the compounds obtained were insoluble in acid, and their nitrogen and hydrogen decreased, in general, as the concentration of alkali increased; thus the substance extracted with 50 % sodium hydroxide contained only 3·84 % hydrogen and 8·98 % nitrogen.

So far from being inert to alkalies the melanin of sheep's wool was thus readily decomposed by boiling with sodium hydroxide in concentrations over 0·2 %, whilst even this concentration decomposed the pigment on continued boiling.

From these results Gortner considered that in all probability many of the melanins described in the literature were really products of the decomposition of melanin and not the pigment as it occurs in the natural material.

The melanin obtained by Abel and Davis from negro skin was left in the form of pigment "granules" (pigment and pigment structure) after the keratin had dissolved in the alkali; and the pigment was freed from the pigment structure by continued treatment with 5 % hydrochloric acid, and subsequent extraction with potassium hydroxide. It then had the composition C = 53·56 %, H = 5·11 %, N = 15·47 %, S = 2·53 %.

The author [Young, 1916] described the preparation of a melanin from the skin of an Australian aboriginal, by treatment with successive portions of boiling 5 % sodium hydroxide, each portion being allowed to act for a short time only. In this preparation no granules were obtained but the pigment was gradually dissolved in the alkali, from which it was obtained by precipitating with acid. The analysis differed from that of the preparation of Abel and Davis as it gave C = 60·12 %, H = 6·70 %, N = 11·89 %.

More recently an opportunity of obtaining more material came to hand, and further preparations were made, the method being modified so that a more dilute alkali ($N/20$) could be employed in the extraction. Pigments were extracted with this alkali from portions of the skin from two Australian aboriginals and a low caste Cingalee.

Preparation.

It was found that if the skin were soaked in boiling water, the outer layer, which contained practically all the pigment, could be readily removed by scraping. This layer (200 g.) was washed with alcohol and ether to remove grease, and was boiled with 100 cc. of $N/20$ sodium hydroxide for one hour under a reflux condenser. It was allowed to settle and the dark liquid decanted and filtered. The process was repeated with successive portions of fresh alkali until, after four extractions, there was only a very small residue. To the four clear filtrates after cooling, hydrochloric acid was added to about $N/3$, whereby the melanin was thrown down as a dark-brown precipitate. It was allowed to settle, the liquid siphoned off and the precipitate repeatedly washed by decantation with $N/3$ hydrochloric acid. The clear washings were siphoned off as much as possible and water and hydrochloric acid added to make the liquid to 500 cc. of $N/20$ hydrochloric acid. The mixture was then heated to boiling. The precipitates from the first two extracts completely dissolved to a deep brown solution; that from the third extract was only partially soluble; whilst the fourth precipitate did not appear to dissolve at all. The solutions were filtered through a fine filter paper (Schleicher and

Schüll's Blue Band), and the first three solutions mixed. In this way the pigment was separated into two fractions, one soluble, the other insoluble in dilute hydrochloric acid. The solution in hydrochloric acid was cooled, and concentrated acid added until the mixture was $N/3$. The melanin was thereby precipitated and was allowed to settle, the liquid siphoned off, and the residue washed several times by decantation with hydrochloric acid of the same strength. The process of dissolving in $N/20$ acid, filtering and reprecipitating was repeated once, the pigment was then again dissolved in the dilute hydrochloric acid, and the solution dialysed until the liquid no longer gave a precipitate with silver nitrate.

This treatment precipitated the pigment, which was then filtered off on a hardened paper, washed with distilled water, then with alcohol, finally with ether and dried. The paper was folded and extracted in a Soxhlet apparatus, successively with carbon disulphide, light petroleum and ether, and was dried at 100°. It was thus obtained as a black powder, and will be referred to as acid-soluble melanin. Before being dried the powder was readily soluble in dilute ($N/20$) sodium hydroxide, and in glacial acetic and concentrated sulphuric acids. It dissolved on warming in dilute ($N/20$) hydrochloric and acetic acids. After drying at 100° it dissolved only with difficulty in boiling dilute alkali, more readily in stronger alkali, and was almost insoluble in dilute hydrochloric acid.

The pigment from the later extractions of the skin, which was insoluble in dilute acid, was also purified by treatment on the filter paper repeatedly with boiling $N/20$ hydrochloric acid. It was then washed repeatedly with water and subsequently dissolved in warm $N/20$ sodium hydroxide. The melanin was precipitated by adding hydrochloric acid in slight excess, washed by decantation until free from acid, filtered and washed with alcohol and ether, and extracted as before with carbon disulphide, light petroleum and ether, and dried at 100°. This powder, after drying, dissolved completely, but only very slowly, in boiling alkalies.

In the above manner both acid-soluble and acid-insoluble preparations were obtained from the three skins.

Analysis of products.

The carbon and hydrogen were determined by burning in a current of oxygen by Dennstedt's method, lead peroxide being employed to keep back any oxides of nitrogen and sulphur. The yields of material were too small for a separate estimation of sulphur, and in some cases the sulphur was therefore estimated at the same time by Dennstedt's method. The quantity of barium sulphate actually weighed was very small, so that the results must only be regarded as approximate. Nitrogen was estimated by Kjeldahl, the heating with sulphuric acid being continued for four hours after the solution had become clear, as recommended by Dakin and Dudley with pyrrole compounds.

In one preparation nitrogen was estimated both by Kjeldahl's and by Dumas' methods with practically identical results.

In all cases before analysis the material was dried to constancy *in vacuo* at 100° over phosphorus pentoxide.

In some preparations which contained a high percentage of ash samples were again treated by suspending in *N*/3 hydrochloric acid in a dialyser, allowing the acid to dialyse away, and washing and drying as before, whereby the ash was very considerably reduced. In such cases the analysis before and after this treatment is given. The percentages are all calculated for the ash-free substances and the results are tabulated below.

	Sol. in dil. acid					Insol. in dil. acid				
	C	H	N	S	Ash	C	H	N	S	Ash
I. Aust. Black I:										
(*a*) ...	56·41	7·33	—	—	7·02	56·03	5·86	—	—	3·51
(*b*) After further treatment to remove inorg. matter	56·33	7·37	12·46	—	2·78	55·92	6·25	—	1·77	2·60
II. Aust. Black II:										
(*a*) Extracted by first portion of alkali	55·49	8·30	13·54	—	0·67	59·87	7·27	10·80	1·66	1·39
(*b*) Extracted subsequently	56·81	6·68	{11·95 / 12·24}	2·49	4·03					
III. Cingalee:										
(*a*) ...	56·01	6·24	—	—	5·47	56·06	6·58	13·27	2·37	3·09
(*b*) After further treatment	56·06	6·71	14·57	2·56	2·68					
Previously found with 5 % alkali	—	—	—	—	—	60·10	6·70	11·89	—	—

In all cases the ash was left as a light brownish powder, only partially soluble in hot hydrochloric acid, and containing a small amount of silica. Estimations of the iron in the ash were made colorimetrically with potassium thiocyanate, and the quantity was found to vary from nothing in one sample, to 7 % of the total ash in another, the latter quantity corresponding to 0·2 % of the original pigment.

The iron was therefore not an essential part of the melanin molecule.

The ash also contained a trace of sulphate.

From the foregoing analyses it is seen that the ash was materially reduced by treatment of the melanin with acid, without altering the carbon and hydrogen percentage calculated on the ash-free substance. It would appear, therefore, that the ash is not an essential part of the melanin.

Action of alkali on the acid-soluble melanin.

A small quantity (about 0·5 g.) of the acid-soluble pigment from the Cingalee skin was boiled with *N*/20 alkali in a reflux apparatus. The sample which had been previously dried at 100° dissolved only slowly, passing, however, completely into solution after about five hours' boiling. The boiling was continued for 15 hours, after which the mixture was cooled, exactly

neutralised with *N* hydrochloric acid, and sufficient acid added to bring the whole to *N*/20. The melanin was partially precipitated, and even when heated to boiling some of the precipitate did not redissolve.

That this was not due to the drying alone was shown by warming another portion of the original dried melanin with the alkali until solution had taken place, neutralising and making to *N*/10 hydrochloric acid and boiling. In this case the melanin completely dissolved.

The pigment soluble in acid behaved, therefore, like Gortner's pigment, in that it was altered slowly by continued boiling with dilute alkali into a substance no longer soluble in dilute acid. Sufficient material was not available to test whether nitrogen and hydrogen were lost during this treatment.

Discussion of Results.

It will be seen from the table of results that variations were found in the percentage composition of the different preparations. The acid-soluble pigment generally contained a lower carbon percentage and a greater percentage of nitrogen and hydrogen than the acid-insoluble pigment subsequently extracted from the same skin, although the insoluble preparations from one skin did not always contain less nitrogen and hydrogen than soluble preparations from another. It is observed also that in the case where the first extraction was kept separate the same difference was found between this and the pigment from subsequent extracts, although both pigments were soluble in acids. Continued boiling with dilute alkali converted the pigment soluble in acid to one insoluble in acid; it seems therefore probable that in extracting the pigment even with very dilute sodium hydroxide (*N*/20) it is gradually decomposed, losing nitrogen and hydrogen and gradually changing to a pigment insoluble in acid.

The melanin of black skin thus resembles that prepared from wool by Gortner in that a pigment may be extracted with alkali which is soluble in dilute acids, and which, on further heating with alkali, changes to a pigment insoluble in dilute acids, with loss of nitrogen and hydrogen. In this case, however, a constant product could not be obtained, and it is possible that none of the preparations represents the pigment as it occurs in the skin.

The preparations gave quite different analyses from the pigment from negro skin prepared by Abel and Davis.

References.

Abel and Davis (1896). *J. Exp. Med.* **1**, 361.
Gortner (1910). *J. Biol. Chem.* **8**, 341.
Young (1914). *Biochem. J.* **8**, 460.

XV. CONSTITUENTS OF THE BARK OF *ZANTHOXYLUM MACROPHYLLUM*, OLIVER.

By JOHN AUGUSTUS GOODSON.

From the Wellcome Chemical Research Laboratories.

(*Received December 24th, 1920.*)

The chemistry of Zanthoxylum species is in a confused state partly owing to the doubtful authenticity of the material examined by various investigators but chiefly due to the confused botanical synonymy of the genus. This difficulty has arisen largely through the transfer by Engler [1895] of a large number of species from Zanthoxylum to the genus Fagara so that closely allied species have been examined under apparently distinct names, with the result that a number of substances have been described as new, which are so similar to substances already known as to warrant the assumption that they are identical.

In the course of the present investigation the author has had occasion to make as complete an examination as possible of the literature relating to the chemistry of Zanthoxylum and Fagara species and as the results present some interesting points they are briefly summarised here. The following list of nine species includes all that have been examined and from which characteristic and well-defined substances have been isolated:

(1) *Zanthoxylum brachyacanthum*, F. Muell (*F. brachyacanthum*, Engler) contains the alkaloids 1-α-canadine methochloride, $C_{21}H_{24}O_4NCl$, and γ-homochelidonine [Jowett and Pyman, 1913].

(2) *Z. Ochroxylon*, D. C. (*F. monophylla*, Lam.) contains two alkaloids; $C_{24}H_{23}O_6N$, and a second for which no formula is given [Leprince, 1911].

(3) *Z. Clava-Herculis*, Linn. (*Z. carolinianum*, Lam.; *F. caroliniana*, Engler). According to Perrins [1862] this contains berberine, and more recently Gordin [1906] has isolated from it xanthoxylin S, $C_{14}H_{12}O_4$ or $C_{21}H_{18}O_6$. There seems to be some doubt as to the authenticity of the bark used by Perrins, and Wehmer suggests that it was really derived from *Z. caribaeum*, Lam., which Perrins himself suggests as a synonym for the species he used though according to the Index Kewensis the two are distinct species.

(4) *Z. senegalense*, D. C. (*F. xanthoxyloides*, Lam.) contains an alkaloid $C_{21}H_{23}O_4N$ or $C_{20}H_{17}O_4N$ and a neutral substance $C_{10}H_{10}O_3$ or $C_{14}H_{14}O_4$ [Giacosa and Soave, 1889], caprinamide, xanthotoxin $C_{12}H_8O_4$, fagarol $C_{20}H_{18}O_6$ [Priess, 1911] and fagaramide $C_{14}H_{17}O_3N$ [Thoms and Thümen, 1911].

(5) *Z. macrophyllum*, Oliver (*F. macrophylla*, Engler) contains fagaramide and lupeol (see below).

(6) *Z. Budrunga*, D. C. (*F. Budrunga*, Roxb.) contains xanthosterin $C_{23}H_{39}OH$ [Dieterle, 1919].

(7) *Z. flavum*, Vahl. (*F. flava*, Kr. et Urb.) contains a lactonic substance $C_{11}H_{10}O_3$ and an ether-lactone $C_{14}H_{12}O_3$ [Auld and Pickles, 1912].

(8) *Z. americanum*, Mill (*Z. fraxineum*, Willd.) contains xanthoxylin N, $C_{15}H_{14}O_4$ [Gordin, 1906].

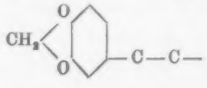
(9) *Z. piperitum*, D. C. contains a crystalline substance $C_{10}H_{12}O_4$ [Stenhouse, 1854, 1857].

The presence of alkaloids has been recorded in all the above mentioned species, but the amount present appears generally to be small except in the four cases (*Z. brachyacanthum*, *Z. Ochroxylon*, *Z. senegalense* and *Z. Clava-Herculis*) in which they have been isolated and characterised. The other definite constituents fall into three classes: lactones, phytosterols and amides.

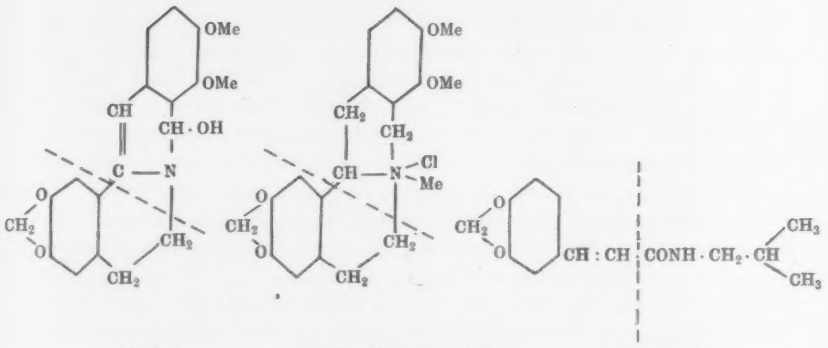
Thoms and Thümen [1911] have suggested that the neutral substance (M.P. 120°) isolated by Giacosa and Soave from *Z. senegalense*, to which they assigned the formula $C_{10}H_{10}O_3$ or $C_{14}H_{14}O_4$ was in all probability fagaramide $C_{14}H_{17}O_3N$. The name xanthoxylin has been applied to three apparently distinct substances two of which have been distinguished by Gordin [1906] as xanthoxylin S and xanthoxylin N. It seems possible that xanthoxylin S from *Z. Clava-Herculis*, for which Gordin proposed the formula $C_{14}H_{12}O_4$ or $C_{21}H_{18}O_6$, is fagaramide, since, although it is not recorded as containing nitrogen, this constituent is not mentioned as having been sought for, and the composition and constants of the substance are otherwise in close accord with those of fagaramide. Xanthoxylin N, $C_{15}H_{14}O_4$ [Gordin, 1906] from *Z. americanum*, Mill and the lactonic substance $C_{11}H_{10}O_3$ [Auld and Pickles, 1912] from *Z. flavum*, Vahl. are similar in properties, and may be identical.

Again the melting points of xanthosterin $C_{23}H_{39}OH$ [Dieterle, 1919] from *Z. Budrunga*, D. C. and its derivatives are in striking agreement with those of lupeol now shown to be present in *Z. macrophyllum*. The analytical figures are not in good agreement, but this is not surprising in view of the difficulty of purifying phytosterols; thus the formula for lupeol is variously given in the literature as $C_{26}H_{42}O$, $C_{30}H_{50}O$, and $C_{31}H_{50}O$, the second being probably correct.

It is interesting to note that fagaramide occurs in the two species *Z. senegalense* and *macrophyllum*, which are both West African, and as noted below are closely allied. A further point of interest lies in the fact that berberine, 1-α-canadine methochloride, and fagaramide which have been isolated from four of the species mentioned above contain the grouping



in common.



Berberine 1-α-Canadine methochloride Fagaramide

Of the nine species examined all but two, viz. *Z. americanum* and *Z. piperitum*, were included in Engler's transfer. No marked difference can be traced between the components—so far as they have been ascertained—of these two species and those found in Engler's Fagara species, so that the chemical evidence appears at present to be of no value in deciding for or against the validity of Engler's view.

Zanthoxylum macrophyllum.

Zanthoxylum macrophyllum, Oliver (*Fagara macrophylla*, Engler) of the natural order Rutaceae is a tree 30 or 40 feet high, with either spreading or ascending prickles on its leaf-stalks, and leaves composed of numerous leaflets 6 to 8 inches long. It is closely related to but quite distinct from *Fagara xanthoxyloides*, Lam. (*Zanthoxylum senegalense*, D. C.); the latter does not exceed 20 feet in height, has recurved prickles and leaves with not more than nine leaflets, 2 to 3½ inches long. Both species occur in Upper Guinea. The present investigation shows that the bark of *Zanthoxylum macrophyllum* contains fagaramide, previously isolated by Thoms and Thümen [1911] from *Fagara xanthoxyloides* and thus further emphasises the close relationship of the two species, already indicated by their structural similarity.

The material used in the present investigation came from the Gold Coast Colony and was kindly supplied by the authorities of the Imperial Institute. The bark is said to be used by the natives as an analgesic, especially in cases of toothache and rheumatism. It produces a tingling sensation in the mouth when chewed.

Experimental.

The material contained 11·2 % of moisture and 4·7 % of ash. Only traces of alkaloid could be detected by the usual reagents. The finely ground bark gave the following percentages of extract on exhaustion in a Soxhlet apparatus with solvents in the order named: petroleum (B.P. 35–60°), 1·46; ether, 0·95;

chloroform, 1·48; alcohol, 12·07; water, 4·80. Of these extracts the first and second appeared to contain all the substance producing the tingling sensation, to which presumably the alleged physiological action is due.

Petroleum extract. The ground bark (32 kilos) was extracted with hot petroleum (B.P. 35°–60°). On standing this deposited a sparingly soluble substance amounting to 60·4 g. or 0·18 % by weight of the bark, which proved to be fagaramide (see below). The mother liquors on concentration deposited in several crops 85·9 g. of a phytosterol, which was shown to be lupeol. The residue of the petroleum extract was of a resinous character and contained the substance producing the tingling sensation when applied to the tongue. Attempts to isolate this substance in a pure state were unsuccessful.

Ether extract. The ground bark after extraction with light petroleum was exhausted with hot ether, the solution shaken with aqueous solution of potassium hydroxide, separated and the solvent distilled off. The residue on extraction with petroleum yielded a further quantity of fagaramide, whilst the resinous active substance remained in solution.

Identification of Fagaramide.

The crude fagaramide, obtained as described above, was repeatedly recrystallised from ethyl acetate, and so obtained in colourless six-sided plates, which softened at 105° and melted at 119·5° (corr.). It gave Gaebel's test [1910] for the methylene di-oxy group, and readily absorbed two atoms of bromine.

	Found	Calculated for fagaramide, $C_{14}H_{17}O_3N$
C	67·8, 67·6	68·0
H	7·0, 6·8	6·9
Mol. weight	231, 241	247·2

Fagaramide is hydrolysed with difficulty by alkalis or acids. It was found most convenient to obtain the acid hydrolytic product, piperonylacrylic acid, by alkaline hydrolysis, and the basic hydrolytic product, *iso*-butylamine, by acid hydrolysis.

Alkali hydrolysis. 0·5 g. of fagaramide was boiled for four hours with a mixture of 12 g. of 50 % aqueous sodium hydroxide and 12 cc. of methyl alcohol. The mixture on dilution with water yielded 0·33 g. of unchanged fagaramide. The filtrate after acidification yielded to ether 0·13 g. of piperonylacrylic acid. The piperonylacrylic acid (from several hydrolyses) was recrystallised from ether, in which, when dry, it is only sparingly soluble, and obtained in almost colourless needles, melting at 243° (corr.). The melting point was not depressed on mixing with piperonylacrylic acid prepared from piperonal. Found C = 62·1, H = 4·2; calculated for piperonylacrylic acid, $C_{10}H_8O_4$, C = 62·5, H = 4·2.

Acid hydrolysis. One g. of fagaramide was heated to 130–140° for 14 hours in a sealed tube with 5 cc. of alcohol, 2 cc. of concentrated hydrochloric acid and 3 cc. of water. The tube was washed out with water and ether, the aqueous

solution separated and evaporated to dryness under reduced pressure, when 0·25 g. of crude *iso*-butylamine hydrochloride was obtained. The latter after purification with animal charcoal and recrystallisation from alcohol melted at 178–179° (corr.). The platinichloride changed colour slightly at 204° (corr.), considerably at 225° (corr.) and melted with frothing at 242–243° (corr.). Found Pt = 36·4; calculated for *iso*-butylamine platinichloride, $(C_4H_{11}NH)_2PtCl_6$, Pt = 36·0.

Contrary to the statement of Thoms and Thümen [1911] fagaramide forms salts, which however are easily dissociated. When a solution of fagaramide in chloroform containing dry hydrogen chloride was evaporated at atmospheric temperature under reduced pressure, a crystalline *hydrochloride* melting at 137° (corr.) was obtained. Found HCl = 6·6; calculated for $(C_{14}H_{17}O_3N)_2HCl$, HCl = 6·9.

Identification of Lupeol.

The crude lupeol obtained as already described was repeatedly recrystallised from ethyl acetate and obtained in colourless needles melting at 216° (corr.), and having a specific rotatory power in chloroform of $[\alpha]_D$ + 31·0° (c = 2·5165, l = 1 dcm., α_D + 0·78°). It gave the usual colour reaction with acetic anhydride and sulphuric acid. Found C = 84·6, 84·6, H = 11·6, 11·9; calculated for $C_{30}H_{50}O$, C = 84·4, H = 11·9.

The following derivatives were prepared:

Acetyllupeol, M.P. 220° (corr.), $[\alpha]_D$ + 44·3° in chloroform (c = 2·5235, l = 1 dcm., α_D + 1·12°), found C = 81·8, H = 11·1; calculated for

$$C_{30}H_{49}O.CO.CH_3,$$

C = 82·0, H = 11·2.

Benzoyllupeol, M.P. 272° (corr.), $[\alpha]_D$ + 62·0° in chloroform (c = 2·5020, l = 1 dcm., α_D + 1·55°), found C = 83·7, H = 10·0; calculated for

$$C_{30}H_{49}O.CO.C_6H_5,$$

C = 83·7, H = 10·3.

Monobromolupeol, M.P. 183° (corr.); found Br = 15·5, calculated for $C_{30}H_{49}OBr$, Br = 15·8.

Monobromo-acetyllupeol, M.P. 232° (corr.).

It was at first thought that the phytosterol obtained from *Z. macrophyllum* was identical with androsterol obtained by Moore [1909] from *Apocynum androsaemifolium*. No direct comparison could be made as the original reference specimens of androsterol and its derivatives were destroyed in the fire which occurred a few years ago at the Brussels Exhibition. The properties of androsterol and lupeol are however so similar that there can be little doubt of their identity. The phytosterol obtained in this investigation, together with the acetyl and benzoyl derivatives, have been directly compared by means of their melting-points with specimens of lupeol, acetyllupeol, and benzoyllupeol kindly supplied by Prof. van Romburgh, and the identity of the two established.

Examination of the active resin.

The resinous residue obtained as already described exhibited in a marked degree the property of causing a tingling sensation on the lips and tongue, shown by the crude bark when chewed. As this resin could not be induced to crystallise and nothing crystalline could be separated from it by steam distillation or other means it was boiled with $N/2$ potassium hydroxide in alcohol, the alcohol distilled off, and the "unsaponifiable matter" extracted with ether. It constituted 24·4 % of the crude resinous substance and still possessed, apparently undiminished, the property of causing intense tingling of the tongue and lips. It was readily soluble in alcohol or ether and insoluble in water or alkalis. As it could not be crystallised an attempt was made to distil it *in vacuo*. It yielded two fractions, boiling at 240–280°/40 mm., and 280–350°/40 mm. respectively. These distillates though both still physiologically active were less so than the crude unsaponifiable matter. Some decomposition evidently occurs towards the end of the distillation as on opening the flask there was a slight smell of ammonia. The lower boiling of the two distillates when dissolved in alcohol and allowed to stand for some days deposited a small amount of a crystalline substance, which after recrystallisation from ethyl acetate melted at 82° and contained nitrogen. This substance did not produce the tingling sensation.

Mr G. S. Boulger, F.L.S., of the Imperial Institute Staff, to whom the author is much indebted for assistance with the botany of the subject, points out that Bentham and Hooker placed the Linnean genus *Fagara* and several others under the genus *Zanthoxylum*, which thus became a group of some 150 species, and that Engler's action in restricting *Zanthoxylum* to some nine species, and restoring the one Linnean genus *Fagara*, thus appears to be a very slight and conservative revision.

In conclusion, the author desires to express his warmest thanks to Dr T. A. Henry for his advice and criticism throughout the course of the work.

REFERENCES.

Auld and Pickles (1912). *J. Chem. Soc.* **101**, 1052.
Dieterle (1919). *Arch. Pharm.* **257**, 260.
Engler (1895). *Engler and Prantl, Pflanzenfamilien*, **4**, 115.
Gaebel (1910). *Arch. Pharm.* **248**, 226.
Giacosa and Soave (1889). *Gazzetta*, **19**, 303.
Gordin (1906). *J. Amer. Chem. Soc.* **28**, 1649.
Jowett and Pyman (1913). *J. Chem. Soc.* **103**, 291.
Leprince (1911). *Bull. Sci. Pharmacol.* **18**, 337.
Moore (1909). *J. Chem. Soc.* **95**, 739.
Perrins (1862). *J. Chem. Soc.* **15**, 339.
Priess (1911). *Ber. pharm. Ges.* **21**, 227.
Stenhouse (1854). *Annalen*, **89**, 257.
—— (1857). *Annalen*, **104**, 236.
Thoms and Thümen (1911). *Ber.* **44**, 3717.

XVI. THE ANTI-SCORBUTIC VALUE OF DRIED MILK.

By HARRY JEPHCOTT and ALFRED LOUIS BACHARACH.

From the Glaxo Research Laboratory, 42 a, *Bravington Road, W.* 9.

(*Received January 1st, 1921.*)

A considerable conflict of evidence exists as to the presence or absence in dried milk of the anti-scorbutic accessory food factor, or vitamin, commonly referred to as "water-soluble *C*." The experiments described in this paper were an attempt to clear up some of the discrepancies.

The animals used were guinea-pigs, and the methods those of previous investigators, except where it seemed to us necessary to make certain modifications by way of additional safeguards.

Care was taken as far as possible to eliminate abnormal animals before experiment. In every case the young animal weighing about 250 g. was put into a cage on a normal diet of green-stuff (cabbage or cauliflower-leaf) with oats and bran, and was only given a milk diet when it showed a normal and steady rate of growth, and had reached 300–350 g. Before the experimental diet was begun, all hay was removed from the cages, and sawdust alone used as bedding.

The normal diet of a guinea-pig contains no moisture other than that associated with the cabbage-leaves or other vegetable matter on which it lives, and we found that, in the absence of green-stuff (as in the case of certain animals that we put on to a purely scorbutic diet), only about 50 cc. of water were taken during the day. Whatever the nature of the food, it is obviously unreasonable to expect animals, whose constitution is only adapted to the taking of a small quantity of liquid, to take several times this quantity and still maintain perfect health. To avoid, therefore, undue dilution of the food the milk-powder was made into a thin cream with *three times its weight of hot water*. We were thus giving our reconstituted milk in a form twice as concentrated as that used by Barnes and Hume [1919], and other investigators.

In order to make the reconstituted milk as palatable as possible to the animals, it was made up freshly every morning and evening, about twice as much being given for the night as for the day. It was reconstituted with hot water and not with cold or boiling water, in order to postpone as far as possible the formation of a layer of solid fat on the surface, which seemed

to be resented by the animals, and also because they appeared to us actually to prefer it with the chill off. Fresh oats and bran were given every morning, but not until after the milk had been put in the cages, in order to prevent the animal satisfying its appetite on the oats and bran, and then feeling disinclination for the milk. Hand-feeding was only necessary in quite a few cases, for the first day or two of an animal's change of diet. The disturbing effect on a guinea-pig of so unusual a diet as milk is only accentuated by so unusual a method of nutrition as hand-feeding with a glass-tube. In all cases sufficient food was given to ensure that some was left over when the next allowance was supplied.

Four series of experiments were undertaken. In the first three, comparison was made of summer milk, winter milk, and winter milk neutralised with sodium bicarbonate before drying. All three were dried by the roller process, in which the milk is in contact with the source of heat for less than three seconds. The fourth variety was full-cream milk dried by the spray process. This was purchased direct from the makers and packed into air-tight tins immediately on arrival. We have no details as to the time for which such milk is heated during manufacture but the remarks made by Hart, Steenbock and Smith [1919], and others, would suggest that it is subject to prolonged heating.

The winter milk was dried by one of us (H. J.) in the first week of December, 1919. The weather was cold and wet, and no cattle were in the fields. The milk when received was perfectly fresh, having an acidity of 17·3° and was immediately dried, sifted, and placed in air-tight tins. The neutralised winter milk was dried at the same time. The acidity of the milk before drying was 26·5° and 11 ozs. of sodium bicarbonate were added to 38 gallons. The summer milk was part of a considerable quantity of milk dried at the same factory in late August and early September. Its acidity before drying was 19·9°, and the powder was stored in hermetically sealed tins, and transferred to smaller air-tight tins for experimental purposes. We give below the composition of the powders:

Table I.

	Winter milk	Summer milk	Neutralised winter milk	Spray process
Moisture	2·6	2·8	3·3	4·3
Ash	5·9	5·8	6·6	5·6
Fat	26·7	27·0	27·2	28·2
Lactose	37·3	36·3	35·4	35·6
Protein	27·0	27·8	26·5	25·6
	99·5	99·7	99·0	99·3

It will be observed that the general composition of these milk powders was almost identical.

Our experiments were begun in January 1920 in the case of the winter and the summer milk series. The experiments in the summer milk series

were completed by the end of May, while the experiments in the winter milk series were concluded at the beginning of July. The experiments in the neutralised milk series were begun in the middle of March and were concluded at the end of July.

It will thus be seen that the winter milk was from two to eight months old during the course of the experiments, while the summer milk was from four to ten months old. The neutralised milk was from three to seven months old; we have no information as to the age of the spray milk.

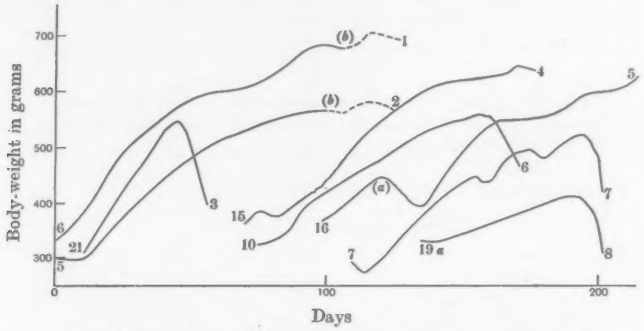


Fig. 1. Diet of oats, bran, and summer dried milk (roller process).

Curves 3, 6, 7 and 8. Death from disease; no scurvy in 7, 10, 21 (Curves 7, 6, 3); slight scurvy in 19 *a* (Curve 8).

Curve 5. This animal (16) hurt its foot at (*a*); its food consumption fell off, and it developed mild scurvy; from this it recovered completely, without any change of diet.

Curves 1 and 2. (*b*) indicates change over to full normal vegetable diet.

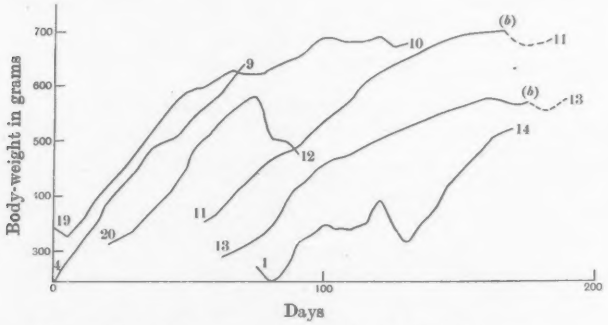


Fig. 2. Diet of oats, bran, and winter dried milk (roller process).

Curve 12. Death from disease; no signs of scurvy. Animals 2, 18, and 20 showed similar curves.

Curve 14. Attack of scurvy repelled without change of diet.

Curves 11 and 13. (*b*) indicates change over to full normal vegetable diet.

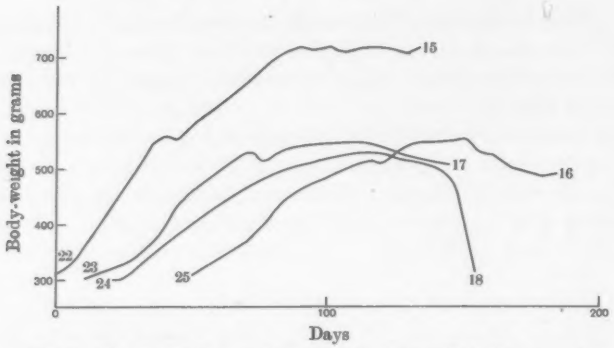


Fig. 3. Diet of oats, bran, and neutralised winter milk (roller process).
Curve 24. This animal died from disease, but post-mortem examination showed many of the characteristics of mild scurvy.

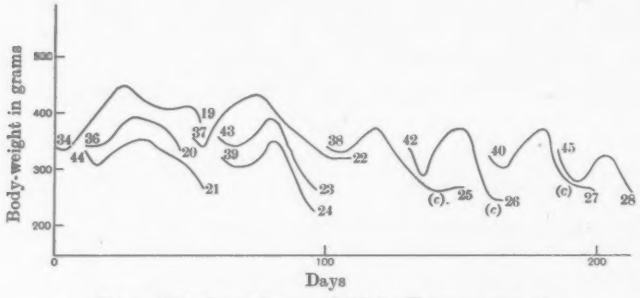


Fig. 4. Diet of oats, bran, and dried milk (spray process).
Curves 25, 26, and 27. (*c*) indicates change of diet to roller process milk. In the case of these three animals (38, 42, and 40) no cure was effected.
Curves for animals 35 and 41, which were cured in this manner, are shown separately (Fig. 5).

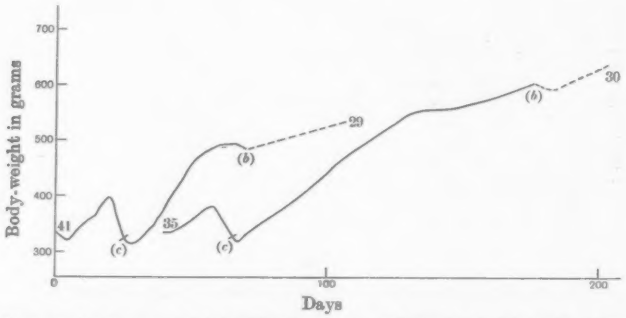


Fig. 5. Diet of oats, bran, and dried milk (spray process) followed by dried milk (roller process).
Curves 29, and 30. (*c*) indicates change from spray process to roller process (summer) dried milk; (*b*) indicates change over to full normal vegetable diet.

The following table and the curves in Figs. 1–5 record the results of feeding groups of animals upon the four varieties of dried milk.

Table II.

Animal no.	Daily consumption in cc., averaged over 30-day period 1.	2.	3.	Length of experiment (days)	Initial weight in g.	Highest weight in g.	Final weight in g.	Remarks
				Series I. Summer milk.				
5	88	145	121	103	308	572	567	No scurvy
6	122	157	124	108	339	692	685	No scurvy
7	90	119	—	82	302	543	420	Death from disease
10	84	108	123	96	330	564	465	Death from disease
15	97	124	136	115	365	633	612	No scurvy. Chloroformed
16	85	122	129	115	368	636	636	No scurvy. Chloroformed
17	85	120	—	77	302	437	332	Slight scurvy. Death from disease
19*a*	73	86	—	65	336	427	311	Slight scurvy. Death from disease
21	128	—	—	47	309	542	370	Death from disease
				Series II. Winter milk.				
1	88	90	160	98	277	530	530	In good health. Chloroformed. Signs of old scurvy
2	107	100	—	73	265	429	421	Died of disease. Slight scurvy in early life
4	117	140	—	73	259	645	645	No sign of scurvy
11	98	143	147	112	546	707	706	No sign of scurvy. Survived
12	88	96	—	71	300	471	308	Slight scurvy. Disease main cause of death
13	91	101	85	112	300	590	577	No scurvy. Chloroformed
18	72	105	—	65	340	392	309	No scurvy. Death from disease
19	117	156	153	129	342	700	683	No scurvy. Chloroformed
20	95	128	—	71	313	604	432	No scurvy. Death from disease
				Series III. Neutralised winter milk.				
22	117	147	165	137	310	732	714	Slight scurvy. Chloroformed
23	86	132	95	98	306	557	505	Mild scurvy. Chloroformed
24	89	107	96	93	306	539	340	Scurvy main cause of death
25	88	142	126	125	306	565	496	Mild scurvy. Chloroformed
				Series IV. Spray process milk.				
34	105	—	—	54	338	470	335	Acute scurvy. Moribund. Chloroformed
36	91	—	—	41	350	399	293	Acute scurvy. Moribund. Chloroformed
37	101	98	—	63	358	451	310	Acute scurvy. Moribund. Chloroformed
38	68	(97)	—	52	338	395	248	Severe scurvy
39	78	—	—	38	333	351	223	Acute scurvy. Moribund. Chloroformed
40	95	—	—	37	299	378	235	Died of acute scurvy[1]
42	92	—	—	35	308	380	244	Died of acute scurvy[1]
43	98	(69)	—	38	317	399	247	Acute scurvy. Chloroformed
44	80	(80)	—	48	332	367	241	Died of acute scurvy
45	70	—	—	29	337	328	233	Died of acute scurvy

[1] See below.

In the case of animals 40 to 45 spray process milk was given at a concentration of 1 in 3 in order to ascertain if the animals could be induced to take sufficient of the milk to avoid scurvy.

Average weight curves are given for each series, as well as the normal growth curve obtained by keeping nine animals on full vegetable diet, but under otherwise the same cage conditions as the animals fed upon milk. The curve thus secured shows, unlike that of Barnes and Hume [1919], a gradual decline in steepness, becoming flatter as the animals grew older. It will be noticed that it is a somewhat steeper curve than any of the others; this is only to be expected, for milk is, at best, an inferior food for an animal with a natural vegetarian diet. Nevertheless, the difference between this normal curve and those obtained for summer and winter dried milk is remarkably small (see Fig. 6).

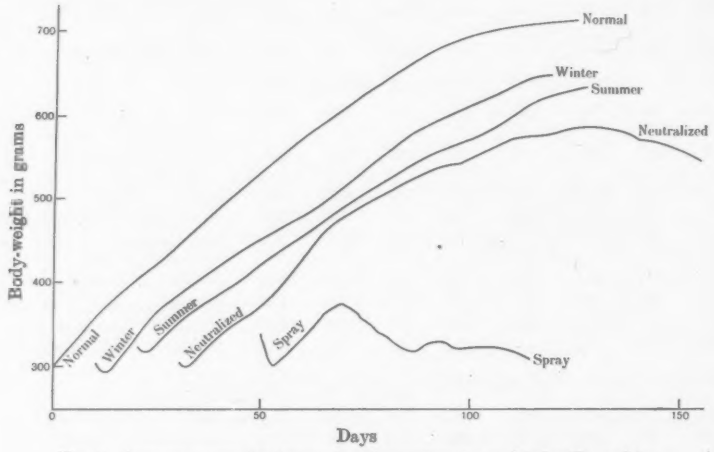


Fig. 6. Average curves for four series of experiments on dried milk, and for a full normal vegetable diet.

It is not considered necessary to give full protocols of all the animals used, but certain of the animals have been of sufficient interest to merit individual comment; we give also individual curves for the majority of animals in each series.

Four animals, two on winter milk (experimental animals 5 and 6) and two on summer milk (experimental animals 11 and 13), after over 100 days on experimental diet had reached a weight that was practically constant, and, to ascertain whether they were full-grown or whether their lack of further growth was due to absence of a sufficient supply of growth-promoting factor, they were then given a normal diet of oats and bran and about 100 g. of cabbage-leaf per diem. No marked growth occurred in any case and the dotted parts of Curves 1, 2, 11 and 13 (Figs. 1 and 2) illustrate this feature.

Certain animals on spray process milk diet were, after they had become definitely scorbutic, placed on summer dried milk (dried by the roller process). In two cases (experimental animals 40 and 42), haemorrhage had already set in, and the animals after a brief rally died (see Table II, Series IV). In two cases, however, a complete cure was effected by the change over from spray process to roller process dried milk.

Table III.

Animal No.	Consumption in cc., average over 30-day periods				Length of experiment (days)	Initial weight in g.	Final weight in g.
35	80[1]	112	142	170	136	330	611
41	103[2]	118	(121)	—	73	330	490

See also Fig. 5.

[1] Change over on 23rd day, weight 364 g.

[2] Change over on 29th day, weight 320 g.

In order to give a general comparison of the anti-scorbutic value of the four milks used, we summarise our results in the following table:

Table IV.

	Summer milk	Winter milk	Neut. milk	Spray milk
Under experiment	9	11	4	12
Developed scurvy	2	3	3	12
Survived, or died of causes other than scurvy	6	9	1	—

Two animals on summer milk which died from disease (probably *coccidium* or *pseudo-tuberculosis*) showed signs of scurvy. One consumed throughout considerably less than the average ration of milk (experimental animal 19 *a*), and the other showed a marked falling-off in its food intake during the three weeks preceding death (experimental animal 17).

Of the three animals on winter milk that showed signs of scurvy, one recovered without change of diet (experimental animal 1), one (experimental animal 12) was weakened by a fall and consequent loss of appetite, and one was recovering from an early attack of scurvy when it succumbed to disease (experimental animal 2). Our conclusion on this point must, therefore, be negative; we have, in fact, found no appreciable difference in anti-scorbutic value between winter and summer dried milk.

When we turn to the actual anti-scorbutic value of dried milk as compared with raw milk, we find considerable discrepancy between our results and those of Barnes and Hume [1919] and Chick, Hume and Skelton [1919]. We are greatly indebted to these workers for the invaluable suggestions contained in their pages, and, as will have been made clear above, have followed their methods very closely. We wish, in conclusion, to point out how we consider this discrepancy has arisen, and how it can be explained.

For purposes of comparison we have expressed the amount of dried milk taken in terms of equivalent cc. of ordinary milk. (Thus, when an animal took 60 cc. of our double-strength concentrated milk containing 15 g. of dried milk and 45 g. of water, we refer to it as having consumed 120 cc. of milk).

It should be observed, in the first place, that up to the publication of a paper by Hart, Steenbock and Ellis [1920] there was no record of any guinea-pig on experimental diet reaching a weight of 500 g.; in most cases, indeed, even 400 g. was not achieved. In the paper of the above mentioned workers a certain advance was made, and out of 54 animals, one reached a weight of over 600 g. and eleven more a weight of over 500 g.

None of our animals when weighing between 300 and 400 g. consumed more than 135 cc. of milk, and in some cases, the average daily consumption was lower than 100 cc. Barnes and Hume [1919, p. 308] state explicitly "that scurvy could be prevented and health maintained in those cases in which 150 cc. (of raw milk) were taken." On the other hand, recent workers [Hart, Steenbock and Ellis, 1920] claim to have obtained complete protection with 50 cc. of fresh summer milk. It seems possible that one source of discrepancy may be the failure to take into account, as well as the amount of food necessary for protection, the size of the animal protected from scurvy.

In very few cases did any of our animals consume appreciably more than 150 cc. of milk, and, even then, only when their weight was well over 500 g. Frequently, at a later period, the consumption actually dropped off; the animal was no longer growing rapidly, and food was mainly needed to maintain metabolism for an animal leading a sedentary life, and not to supply the matter or the energy for building new tissue. Even so, with this smaller intake, the animals still remained free from scurvy.

It would appear, therefore, that on the average growing guinea-pigs can find in milk reconstituted from dried milk as much protection as, according to Barnes and Hume, is afforded by raw milk. It is desirable, if possible, to gain some idea as to what is the minimum of such reconstituted milk required for protection. We have adopted the following method for arriving at this quantity. Ignoring those animals that showed signs of scurvy, since protection in their case was not complete, and eliminating, in the case of those that died from other causes, that part of their career where they were eating little and losing weight (to include those parts would, obviously, quite inaccurately lower the estimated minimum protective dose of milk), we have then divided the time of normal growth into 8-day periods, and for each 8-day period we have calculated the average weight of the animal (W) and the average consumption of milk in cc. (V). The expression $\frac{100V}{W}$ represents the average daily consumption per 100 g. body-weight. This has been calculated for each animal and the average for the two series where there was complete protection from scurvy is also taken. We give below a summary of the results:

Table V.

Animal No.		Period (days)	Minimum $\frac{100V}{W}$	Maximum $\frac{100V}{W}$	Average $\frac{100V}{W}$
1	Winter milk	4– 27	32	36	33
4	„ „	4– 73	24	37	29
11	„ „	1–104	17	28	23
13	„ „	1– 96	19	27	22
18	„ „	4– 51	20	35	23
19	„ „	4– 67	24	33	29
20	„ „	3– 54	20	31	26
	Average for winter milk series $\frac{100V}{W}=27.$				
5	Summer milk	10–103	22	35	27
6	„ „	3–106	19	31	25
7	„ „	3– 88	22	32	27
10	„ „	6– 85	21	26	23
15	„ „	1– 96	20	34	26
16	„ „	35– 96	21	30	25
17	„ „	3– 70	19	32	26
19a	„ „	4– 59	23	20	25
21	„ „	1– 36	29	33	32
	Average for summer milk series $\frac{100V}{W}=26.$				
	Average for two series (16 animals) $\frac{100V}{W}=26.$				

It will be seen from these figures that the average daily intake of milk in cc. per 100 g. body-weight varies considerably. There is variation in the case of each animal for different 8-day periods in its history; there is variation between the general average of one animal and another. The averages, for the two series, are identical within the degree of accuracy possible for this kind of experiment. The average for the whole series indicates that the minimum protective dose of milk reconstituted from dried milk for a guinea-pig of 400 g. body-weight is about 110 cc.

It has previously been stated [Harden and Zilva, 1918] that alkali has a destructive effect upon vitamins and the results obtained with neutralised milk would indicate that there is some destruction of the anti-scorbutic vitamin by alkali in the quantities used in this experiment. The reduction of the anti-scorbutic value was, however, slight and in no case were symptoms of scurvy observed in less than 100 days; even then they only appeared when rather less than the average quantity of raw milk required for protection was taken.

In series IV, even when animals which took small quantities of milk are eliminated, it can only be concluded that the anti-scorbutic factor had been very considerably affected, even if not entirely destroyed. A description of the spray process, from which it would appear that the milk is held at high temperatures for a very considerable time, is given by Hart, Steenbock and Smith [1919]. It has been suggested by these workers and others [*e.g.* Delf, 1920] that heat is responsible for diminution in the anti-scorbutic value of certain cooked or manufactured foods. Recent work, however, suggests that

oxidation may be an important factor in destroying the anti-scorbutic vitamin [Zilva, 1920].

That winter milk should be found to have as high anti-scorbutic value as summer milk is contrary to the opinions generally held, and to certain other experiments, but enquiry has shown that the cattle were being partly fed upon swedes at the time when the winter milk was obtained, and it is probable that the anti-scorbutic value was due to this cause.

There is some evidence to show that the method of drying may have a considerable influence on the degree to which the anti-scorbutic vitamin in milk is affected. The milks with which we have experimented were subjected to the high temperature of drying for not more than $2\frac{1}{2}$ seconds and, when the milk left the rollers, it was still steaming. This clearly indicates that there was considerable moisture present when the milk ceased to be in contact with the hot surfaces, and its temperature cannot, therefore, have appreciably exceeded 100° at any time. The comparatively low temperature of drying, the short period of contact with the hot rollers, and the relatively short exposure while hot to oxidation (compared with the spray process), may all contribute to the results we have found.

We are aware that under unsuitable conditions of storage, especially exposure to the air, dried milk undergoes marked change, and it is possible that the accessory food factors present are affected. This is a purely theoretical conclusion based upon physical changes in dried milk, and we have not, at present, any experimental evidence to support this view. Other workers [Hess and Unger, 1919] have expressed similar opinions.

We have called attention earlier in this paper to the fact that milk, especially full-cream milk with its high percentage of fat, is hardly a suitable diet for guinea-pigs. The question, therefore, arises how far such experiments are applicable to infant feeding. It has been argued that pasture-fed cattle yield milk of higher anti-scorbutic value than do stall-fed animals. It might equally be argued that human milk would, for the same reason, be markedly deficient in anti-scorbutic value, as compared with cow's milk. Whether or not this is the case cannot be known until experiments have been made with human milk.

We can only conclude from our own work that the results give a considerable measure of support to the clinical opinion, widely held in places where dried milk has been used for infant feeding during many years, that perfectly fresh milk dried by the roller process and stored in air-tight vessels contains sufficient anti-scorbutic accessory food factor to prevent scurvy in infants fed solely on a milk diet [*e.g.* Coutts, 1918].

It should be recognised, however, that whilst such dried milk is apparently equal to raw milk in anti-scorbutic value neither is rich in anti-scorbutic vitamin and where infants are only taking small quantities of milk or are upon a diet which is mainly farinaceous, the addition of a little fruit-juice is not only desirable but essential.

SUMMARY.

Animals have been fed upon summer milk, winter milk, and neutralised milk dried by a roller process and upon milk dried by the spray process. In the case of the summer and winter milks the anti-scorbutic values were found to be about equal to one another and to those of the original raw milks. The neutralised milk had an anti-scorbutic value slightly less than that of the raw milk and the spray process dried milk was markedly deficient in anti-scorbutic value.

Of the summer and winter dried milks a daily dose of 26 cc. per 100 g. body-weight, of reconstituted milk was, on the average, sufficient to afford protection against scurvy in the guinea-pig.

REFERENCES.

Barnes and Hume (1919). *Biochem. J.* **13**, 306.
Chick, Hume and Skelton (1919). *Biochem. J.* **12**, 131.
Coutts (1918). Reports to the Local Government Board on Public Health and Medical Subjects. New Series, No. 116, Part I, 100.
Delf (1920). *Biochem. J.* **14**, 211.
Harden and Zilva (1918). *Biochem. J.* **12**, 259.
Hart, Steenbock and Ellis (1920). *J. Biol. Chem.* **42**, 383.
Hart, Steenbock and Smith (1919). *J. Biol. Chem.* **38**, 312.
Hess and Unger (1919). *Amer. J. Dis. Child.* **17**, 221.
Zilva (1920). *Biochem. J.* **14**, 740.

XVII. THE EFFECT OF DIET ON MAMMARY SECRETION.

By GLADYS ANNIE HARTWELL.

From the Physiological Laboratory, Household and Social Science Department, King's College for Women, Kensington, London.

Thesis approved for the Degree of Master of Science in the University of London.

(*Received December 22nd, 1920.*)

I. Introduction.

The literature of research work performed to elucidate the effect of diet on the secretion of milk does not give the impression that there is a definite relation between the two things. On the other hand, the great experience of the farmer indicates how closely these are related, and any precise information would be not only of value to him, but might also assist in overcoming the disability so frequently met with in modern women, many of whom are unable to breast-feed their children owing to a deficient secretion.

Various methods are available for studying this problem.

1. The animals could be fed on diets of well-known foods, used in different proportions, such as bread, meat, butter, etc.

2. Synthetic diets could be employed, consisting of isolated carbohydrates, proteins, fats, vitamines, salts and water.

3. A combination of both the above methods is possible.

It seemed probable that some factors could be most easily found in one way and some in another.

Since man lives on natural foods and not on a diet of synthetic foodstuffs, the first method is more likely to yield results, directly applicable to human mothers and babies, than either of the others. Hence in the experiments described in this paper, the diets were chosen from ordinary foods and the rate of growth of the young during the lactation period was gauged by the daily increase in weight. The mother's weights were also recorded, since an ideal diet would keep up the mother's weight, and at the same time provide for the rapid growth of her young, while an inadequate diet might fail in one or both respects.

An omnivore had to be used and rats were chosen, because they readily eat all foods normally taken by man, and psychic effects resulting from forcing an animal to eat unpalatable food were prevented. Also rats were

most convenient, since they are small, have short gestation and lactation periods and will breed throughout the year, provided they are kept warm. One rat will give birth to and rear as many as six healthy litters in a year.

The drawback to work on rats is that it is difficult, if not impossible, to differentiate whether the results obtained are due to alteration in quantity or quality of the milk.

Although very little appears to be known about the actual factors concerned in the production of milk, it is generally accepted that a nursing mother should have a large proportion of protein in her diet. The experiments described later tend to show that this statement needs modifying to some extent. Up to a certain limit protein is certainly beneficial, it not only promotes good growth in the young, but also helps to maintain the mother's weight and prevent excessive loss, but above that limit, decidedly harmful effects are obtained and the baby rats usually die, showing very typical symptoms.

The experiments of McCollum, Simmonds and Pitz [1916] were made on similar lines to those described in this paper, but the mothers were fed on synthetic diets. These observers showed that a maximum rate of growth in the baby rats can only be obtained when the mother is receiving a full diet, and that with a poor diet, although the litter does gain, yet the gain is not normal, and is at the expense of the mother's own tissues, as shown by the fact that her weight goes down during the lactation period. They further pointed out that there are limits beyond which the mother cannot meet the demands on her tissues and certain factors (especially fat-soluble *A* and water-soluble *B*) must be present in the diet and cannot otherwise be supplied by the mother (or only to a very slight extent for a limited time). On the whole the experiments described below agree with those of McCollum, Simmonds and Pitz, but it is shown here that the loss in weight of the mother, which is so characteristically noticed with a poor diet, can be prevented, except for an initial loss, by adding extractives to the mother's diet, in spite of the fact that it may still be inadequate in other respects. In speaking of the work of these observers one comment suggests itself with regard to the duration of their experiments. Their curves extend over a period of 24 days, but a baby rat usually begins to eat at about the 18th day and is quite capable of living without its mother from the 21st day, so that the last part of the curve may not be entirely due to the nourishment obtained from the mother.

On the other hand the babies might have been prevented from eating for themselves, but this would hardly be working under normal conditions and would put an extra strain on the mother rat.

Hart, Nelson and Pitz [1918] proved that the mammary gland has not the power to synthesise lysine, and that as far as proteins are concerned, the milk secretion is ultimately dependent upon the quality and quantity of amino-acids ingested in the food. The actual dependence of the milk secretion on the food ingested is also noticeable in the experiments described later,

in which the diet is changed during the lactation period, and an immediate effect on the rate of growth of the young can be seen.

These observers further state that the average time taken by a young rat to double its birth weight is 14 days, but they do not support their statement by experimental evidence.

On the other hand McCollum, Simmonds and Pitz show by actual weight curves that the time taken is much shorter, viz. 7 days with a full diet and 8–9 days on a deficient diet. This latter fact is in accordance with results of experiments described below, which are also based on actual figures. Therefore it seems permissible to say that the time taken to double the weight of a new-born rat is 7–9 days and not 14 as stated by Hart, Nelson and Pitz.

A great deal of work has been done on the composition of milk as varied by different diets, but in all cases larger animals were used and consequently a certain quantity of milk could be obtained and analysed. In experiments such as are described in this paper, it is of course impossible to analyse the milk, and its quality can only be surmised from the rate of growth of the young.

Voit's experiments on a bitch showed that the composition of the milk secretion only varied slightly when the mother received different diets, such as meat alone, meat and starch, meat and fat, starch alone, fat alone and also when she was starved. However the composition of the milk did vary to a slight extent and this variation might possibly have been greater had the animal been fed on the same diet for more than 2–3 days.

Lusk [1917] fed a goat on a diet of hay, cornmeal and bran, then starved it for two days, after which it was fed again on the original diet. He found that when the animal was starving the fat content of its milk was greater than normal and nearer in quantity to that found in the milk of the dog. When the goat received its normal diet again, the fat content of the milk was reduced.

It is somewhat difficult to correlate experiments such as are described in this paper with known facts about dairy feeding, because the object in view is different. When feeding animals on the commercial scale, it is necessary to consider what foods produce the greatest quantity of milk and butter fat for the least cost. Although the amount of butter fat and the amount of protein in the milk are generally closely related, at any rate in some animals, yet this may not always be the case.

Again it is difficult to draw conclusions with regard to human milk secretion from results obtained by feeding cows, since the metabolism of a herbivore must be different from that of an omnivore.

It seems quite possible that a milk which is highly suitable for cheese or butter-making might not be so suitable for rearing the young.

In order to obtain an abundant milk secretion, farmers have found it necessary to consider the palatability of the food and this factor has probably the same significance with all animals. The experiments described later show

that in many cases the young grow better when their mother eats more of any given food and therefore the question of palatability must certainly have a bearing on this question.

Eckles [1911] states that in cows it is almost impossible to restore the milk flow to its original amount after once allowing it to run down from lack of food. With rats, however, it appears that a good diet after a poor one succeeds in pulling up the milk secretion practically to a maximum.

Eckles also points out that with cows, if the ration is changed suddenly from grain to grass, a peculiar taste is noticed in the milk, but this is scarcely detected if the ration is changed by degrees; in other words, the milk supply seems to be dependent, to some extent, on the food taken at the time. This fact is also shown by altering the diet of the rat, as a result of which a difference is at once evident in the growth curve of the young.

Eckles further states that for a heavy-milking cow, the food must be concentrated, since sufficient grass to provide material for the secretion cannot be eaten and, therefore, grain must be supplied in addition.

On the whole farmers think that diets rich in protein are the best for getting a good supply of milk and this is certainly supported by the experiments done on rats, provided the ration does not contain a large excess of protein.

A well-balanced diet is recommended for cows and it is evident from the experiments described later that this is an important factor. It is found with rats that butter may have practically no effect when fed with bread only, but may be quite beneficial if fed with a better, more mixed type of diet.

A heavy milking cow usually loses weight for 2–3 weeks (and sometimes more) after the birth of the calf, probably because she is not taking enough food for all her requirements. This feature is also exhibited in the curves shown later. Many of the rats lost a considerable amount of weight for the first few days after the birth of the litter, and for about 48 hours after the birth they rarely ate more than the average rat.

This loss of weight of the mothers may be due to one of the following factors:

1. The energy of the diet might be insufficient to supply food for the young and to replace the mother's heat loss.

2. The diet might be adequate from the energy standpoint, but lacking in certain necessary constituents, which are then supplied from the mother's own tissues, thus causing a loss of weight.

This latter supposition is supported by an experiment of Hart and Humphrey [1915]. They fed a cow on a food with a "nutritive ration" of 1 : 8, *i.e.* one part of protein to eight parts of carbohydrate and fat. Provided the protein given was milk protein, this diet gave a positive nitrogen balance, but if wheat protein was substituted a negative nitrogen balance resulted. The amount of protein in the milk was unchanged and the loss of maternal nitrogen must have resulted from tissue breakdown, consequent on the lower biological status of wheat protein as compared with milk protein.

II. Methods employed.

The actual experiment was started as soon as the litter was born. In the majority of cases the babies were born in the night, but a litter born during the day was not weighed until the next morning, because it was found desirable not to disturb the mother too soon.

During the gestation period the females were fed on an ordinary mixed diet, consisting chiefly of bread, with small amounts of milk and green and other vegetables. They were removed from the males a few days before the birth of the litter. The number in each litter was always reduced to six. The mothers and litters were weighed every day, as nearly as possible at the same time and after weighing they were given their 24 hours' rations. It was impossible to give definite and equal amounts to the various rats, because their appetites exhibited great differences, and what was ample for one rat proved to be starvation diet for another. Hence the method was adopted of keeping the proportions of the constituents of the food constant and allowing the rats to eat what they liked, the amount eaten, of course, being noted. If a rat ate all its food, then the diet was increased the next day. In this way, with a little experience it was easy to gauge the animals' appetites, and feed them so that only small quantities were left each day. It seemed essential that they should have sufficient to satisfy them, otherwise abnormal curves were obtained for the litters, even though the mother was given a very good diet, such as bread and milk (which will later be shown to be one of the best diets for a nursing rat).

Bread alone was taken as the poorest type of diet. In every case, except where 100 cc. milk were given, the animals were allowed water to drink.

It was found that 15·0 g. bread was enough to start with and the amount was increased as necessary. When other foods were to be given, they were mixed with the bread, so that an even mixture was obtained. The bread etc., was always slightly moistened with warm water, because the animals would not eat much dry food, and it was also advantageous because they did not scatter the paste thus made about their cages, as they did dry food. The bread ration was increased by 5·0 g. per diem when necessary and the other foods in corresponding proportions.

The chief diets used were:

1.	Bread only	with water to drink.
2.	„ and meat extract	
3.	„ „ butter	
4.	„ „ dextrin	
5.	„ „ caseinogen	
6.	„ „ milk	
7.	„ „ meat	
8.	Meat only	

Various combinations of the above were also made, *e.g.* bread, butter and meat extract.

The day on which the babies were first seen eating, generally the 18th, is indicated by * on the curves. The mothers were removed on the 21st day, unless the babies did not eat at the usual time, in which case the mother was allowed to remain three days after the babies began to eat.

III. Effect of variation in the mother's diet on the growth of the litter.

(*a*) *General conditions.*

Considerable differences were shown in the weights of the various litters. In some cases the average gain per day was about 4–5 g., while in others it was as much as 11–12 g. It might be expected that the daily gain would be greater towards the end of the lactation period than at the beginning, but this was not always the case. On a good full diet, *e.g.* bread and milk, there was a slight actual increase in weight gained per day towards the end of the lactation period, tending to keep the percentage increase constant, but with a poor diet, *e.g.* bread only, the actual increase in weight per day was usually greater at the beginning than at the end of the period. This latter fact seems what would be expected because such a diet is lacking in certain constituents which are normally present in milk and, it may be inferred, were present in the milk of these rats, as the litters were normally healthy, but small. The constituents are probably supplied from the mother's tissues. Therefore towards the end of the lactation period it is probable that the available reserves of these special substances are coming to an end and consequently the babies do not gain as much as previously. In keeping with this explanation is the fact shown in the curves that the mothers themselves lose weight on poor diets. On other poor diets and especially those rich in extractives there seems to be a fairly uniform daily gain in the weights of the litters and the mothers' weights remain constant.

There are a few remarks which apply generally to the curves. Later they will be treated separately.

1. *The relation of the milk secretion to the amount of food taken by the mother.*

In the majority of cases, given any definite diet, a litter belonging to a mother which ate considerably more than another, did better than the litter of a mother which ate less.

2. *Relation of milk secretion and age and weight of mother.*

The age and weight of the mother seem to be unimportant factors. In some instances the heavier rats brought up their litters better, *i.e.* curves of the young were better but this was by no means a general rule. In some cases a first litter did not grow as rapidly as the later ones, but this again is not always true.

3. *Condition of litters.*

(*a*) *At birth.* Litters of very low weight *i.e.* about 20·0 g. for six (normal weight of six is about 30·0 g.) were generally weakly, but with a good mother they usually pulled up after a few days In a few cases the six weighed a small amount and yet did well. This was when they belonged to exceptionally large litters and this fact alone accounted for their low weight. Also such babies seemed physically fit, while weakly litters were easily detected by their general appearance as well as low weight.

(*b*) *During lactation.* In all cases (except where excess protein in the form of caseinogen was given) the litters were healthy and the babies quite strong and able to look after themselves when separated from their mothers, but the large differences in weight were parallelled by differences in size, *e.g.* a 10–11 days' "bread and milk" baby was often about the size of a 20–21 days' "bread" baby. With the poorer diets, especially bread only, bread and butter, bread and dextrin, the babies' coats were rather thin, more like an animal's summer coat. An exception to this statement was found in the case of bread and meat extract where the babies had a normal coat, rather thick and silky. All the babies (except when the mother was given excess protein, as before mentioned) were able to run about at the usual age. Those belonging to the less well-fed mothers were, if anything, more lively than the fatter litters of the well-fed mothers. The babies belonging to the rats fed on (1) meat extract and bread, (2) meat extract, bread and butter were specially active. The general good health and activity of the young rats, even when the mother's diet is deficient and their own weight small, indicate that it is the quantity rather than the quality of the milk which suffers as the result of defective feeding in the mother.

(*b*) *Individual diets.*

The majority of the figures consist of two sets of curves:

1. The mothers' weights.
2. The weights of the litters.

The corresponding mother and babies are indicated by the same symbols. At least three experiments were made with each diet, and an average growth curve given in Fig. 6.

1. *Bread only.* Basal diet. Water to drink.

The diet consisted of white bread, no crust being used. It was slightly moistened with water to prevent it from getting too dry. An initial ration of 15·0 g. per diem was given and this was increased by 5·0 g. at a time when necessary.

In each experiment the mothers lost a considerable amount of weight and their curves show a varied loss throughout the lactation period. A large initial drop in weight was shown by two of the three animals and this is typical of many other experiments.

The litters show a steady but not large daily increase, which gradually gets less towards the end of the lactation period. Roughly, the curve might be divided into three parts, the first showing a daily increase of about 4·5 g., the second of 3·6 g., and the third of 2·2 g., after which the babies began to eat for themselves (see Fig. 6 and table on p. 158).

This diet consisted chiefly of carbohydrate in the form of starch and protein. It was, therefore, partially deficient in fat, fat-soluble *A*, water-soluble *B*, and the anti-scorbutic factor. Since the young rats were healthy throughout and showed normal activity, it is probable that the mother can supply these deficiencies from her tissues. And, also, as the daily gain gradually becomes less, it might be inferred that the mother's tissue supplies cannot entirely meet the demands made on them.

It is seen from the figures given below that *I* ate more than either of the other rats and her litter showed the best growth; further, *L* ate more than *Y* and the *L* babies did better than the *Y* babies.

I ate 520 g. bread in 17 days
L „ 500 „ „ 19 „
Y „ 370 „ „ 18 „

Result. The rat can bring up a healthy litter, normal in all respects except size, on a diet deficient in fat and vitamines. The mothers lose weight throughout the lactation period (see tables on p. 158).

2. *Effect of adding meat extract to basal diet.* Water to drink.

The initial diet consisted of 15·0 g. bread and 2·0 g. meat extract (a commercial preparation "Jardox" was used). The extract was dissolved in hot water, poured over the bread and an even mixture made. When necessary the diet was increased by 5·0 g. bread and a proportionate amount of meat extract added. This diet is slightly better than the basal diet, but is again deficient in fat and probably in vitamines as well. Nevertheless there are remarkable differences in the results.

The most noticeable feature when meat extract is given, is that the mothers' weights show very little or no loss, and in one case a decided gain was exhibited throughout the lactation period. Also the initial loss in the mothers' weights (pointed out previously when discussing the bread diet) is absent in two of the three animals. In addition the mothers show a steadier weight curve than that usually obtained, when the animals frequently lose or gain about 10·0 g. per day.

The greater increase in growth of the litter as a result of the mother's eating more is again illustrated.

3 ate 490 g. bread and 66 g. meat extract in 18 days
6 „ 490 „ „ 66 „ „ 18
W „ 460 „ „ 74 „ „ 17

The litters show a more even increase in rate of growth throughout the lactation period and do not exhibit the slower growth towards the end of the

period, as shown by litters whose mothers received only bread (see Fig. 6 and table on p. 158).

Result. When extractives are added to the basal diet, the mothers lose very little, if any, weight and their curves are much more regular. As compared with bread diet the milk secretion is slightly improved, more especially at the end of lactation and the litters gain practically equal amounts throughout the lactation period.

3. *The effect of additional carbohydrate in the diet.* Water to drink.

The initial ration consisted of the usual 15·0 g. bread to which 5·0 g. dextrin were added. The dextrin was dissolved in a small quantity of hot water, the bread added and an even mixture made.

The curves representing the rate of growth of the litters are practically identical with those of litters whose mothers fed on bread only. From this it appears that an excess of carbohydrate in the diet makes no difference to the milk supply of the mother, which is very different from the results obtained when excess protein was given to the mother (see curves in Figs. 1, 2, 3 to be described later).

The mothers' curves also are very similar to those of the rats fed on bread only, a typical loss in weight being again a characteristic (see table on p. 158).

Result. Additional carbohydrate in the form of dextrin has no effect on the milk secretion.

4. *Effect of adding fat and fat-soluble A to basal diet.* Water to drink.

Two types of diet were used in these experiments:

(*a*) 15·0 g. bread, and 2·0 g. butter, for initial diet.

(*b*) ,, ,, 5·0 g. ,, ,,

The bread was moistened with hot water, the butter melted and an even mixture made.

The curves of the growth of the litters are again similar to those obtained when the mothers were given a bread diet.

From the experiments made under these conditions, it seems that the extra fat has a slightly depressing effect on the curves of the litters, but this may be an indirect effect, because it was found that with a fatty diet of any kind, the mothers tended to eat less than the normal amount. In such a diet the calorific value was not appreciably depleted, but the animals were getting less protein.

These experiments suggest that fat is an unimportant factor in the question of milk production.

The curves of Fig. 2 suggest that fat may play a more important part if more protein is given, because litters *K* and *M* in the upward parts of their curves did better than litter *O*, the mother in this case receiving no fat in her diet. The significance of this is by no means clear and the point awaits further investigation.

Result. When fat and fat-soluble *A* are added to the basal diet, the mothers lose in weight as on the basal diet and the rate of growth of the litters is practically the same as when the mothers are fed on bread alone (table, p. 158).

5. *The effect of adding milk to the basal diet.*

The milk ration was kept constant at 100 cc. per diem. An initial amount of 15·0 g. bread was given and increased by 5·0 g. as in previous experiments. This is decidedly the best diet used as it contains all the necessary factors. The daily gain of the litter exceeded that obtained with any other diet (see Fig. 6, curve *D*). When milk was given to the mothers it was noticeable that the actual daily increase in weight of the litters became greater towards the end of the lactation period, the exact opposite of what was found with poor diets, *e.g.* bread, or bread and dextrin, or bread and butter. The mothers' curves were somewhat irregular but speaking generally, a loss in weight was followed by a gain, so that there was no very great permanent loss.

Result. Milk added to the basal diet forms a most efficient food and the litters show maximum gains in weight (table, p. 158).

6. (*a*) *Effect of adding meat to basal diet.* Water to drink.

Equal weights of bread and meat were given. The bread was moistened with water as before, the meat minced finely (after removal of fat) and an even mixture made.

The mothers' curves are characterised by gain rather than by loss of weight. The litters gained practically equal amounts daily throughout the lactation period. This result is similar to that described with respect to those litters whose mothers were fed on bread and meat extract. The addition of meat to the bread had an obviously beneficial effect on the secretion of milk (see Fig. 6, curve 2).

Result. Meat added to the basal diet of bread promoted rapid growth of the young, but not so marked as when the mothers were fed on bread and milk (table, p. 158).

(*b*) *Effect of feeding with lean meat only.* Water to drink.

This diet was not so good as that of bread and meat, possibly because the carbohydrate was insufficient for the effective metabolism of the protein taken. The mothers lost weight and the daily gain of the litters was less than half that of the litters whose mothers received bread and meat (see Fig. 6, curve 10 and tables on p. 158).

When fed on bread and meat or meat only the rats ate large quantities, *e.g.* 30·0 g. meat plus 30·0 g. bread, and 60·0 g. meat. These are exceptionally large amounts considering that the rats were by no means fully grown and only weighed 190·0 and 150·0 g. respectively. Even then, when eating meat only, the rats would not be getting an energy value equivalent to that of the

basal diet. The average amount eaten per day on a meat diet was 45·0 g. and on a bread diet the average consumption was about 28·0 g. per day. The energy value of 45·0 g. meat is about 460·0, while that of 28·0 g. bread is 690·0. Hence on a meat diet the mother may not be able to eat sufficient food to get the necessary amount of energy for a good milk secretion.

Result. Meat only is not a diet suitable for producing a good mammary secretion, possibly because the energy of the food is too little to allow for the supply of a good milk secretion.

(c) *The effect of adding bread to a diet consisting entirely of meat.* Water to drink.

A rat was fed entirely on meat for nine days of the lactation period and then her diet was changed to an equal mixture of bread and meat. When the mother was eating meat alone, the litter gained an average of 4·0 g. per day, but when her diet was altered they gained on an average 6·8 g. per day. She lost less weight on the mixed diet and after a few days even showed a tendency to put on weight. The question of change of diet during the lactation period is discussed more fully later.

7. *The effect of adding butter and meat extract to basal diet.* Water to drink.

The proportions used were 15·0 g. bread, 2·0 g. butter and 2·0 g. meat extract (commercial preparation "Jardox"). The meat extract was dissolved in sufficient hot water to moisten the bread. The butter was melted and the whole made into an even mixture.

An interesting point arises here in connection with the mothers' weights. In other experiments, where meat extractives were fed, the mothers did not lose weight to any appreciable degree, but either maintained or slightly increased their original weight. In these earlier described experiments, no fat was given, while in the ones now being discussed, two or more g. of fat were taken per day. The action of the fat appears to inhibit that of the extractives since the mothers lost weight to about the same extent as when they were fed on the basal diet of bread only. A further point is brought out by these results. One mother lost more weight than either of the others which received the same diet. At the same time her litter gave a better growth curve, the improvement being greater than could be accounted for by the extra food eaten by their mother. Similar results were obtained in other experiments, which might be taken to indicate a connection between loss of weight in the mother and gain of weight in the young.

However, the experimental evidence is insufficient to allow of a definite statement either with regard to the metabolism of simultaneously ingested butter and extractives, or the mutual relation between the weights of the mother and her offspring.

Result. When fat is added to a diet of bread and meat extract it does not have any marked stimulating effect on the production of milk and the litters

do not show any great increase in weight (table, p. 158). On the other hand the addition of fat now brings about loss in the mother's weight, which is not evident in its absence (table, p. 158).

8. *The effect of excess protein in the mother's diet* (Figs. 1, 2, 3).

The protein employed was pure caseinogen used in various diets described below.

The results were striking, the litters did well for a time, but eventually died, showing typical symptoms. In most cases they gained weight normally at first, then ceased to gain, finally lost weight and died. The first indication that something was wrong with the babies was that they became very excitable, and when taken out to be weighed, kicked and struggled, whereas normal babies would have kept fairly still. The age at which the symptoms developed varied in the different litters according to the diet chosen, but all exhibited them sooner or later. About two days after the excitability was first noticed, the babies usually showed signs of inability to crawl normally and had a marked tendency to drag one hind leg and roll over towards that side.

Later extensor and contractor spasms were evident and in very bad stages the young rats dashed madly round their cages, bit each other and their mother and knocked themselves wildly against the sides of the cage. The fits generally lasted 3–5 minutes, after which the babies were exhausted and slept.

If the symptoms were not really bad until the babies were about 18 days old, they frequently recovered, if they ate for themselves. When the babies died at about 15–16 days, death was always preceded by extreme weakness and they had the appearance of being starved, although starvation alone could not cause these symptoms. On examination their alimentary canals were found to be practically empty, which naturally suggested interference with the milk supply of the mother. Accordingly the mother was killed and the mammary glands were found to contain no milk, but only a small quantity of a lymph-like substance, while the glands of a well-fed mother contained a large amount of milk, which oozed out directly the tissue was cut. Thus it appears that excess protein in the diet first causes the production of abnormal milk and then stops the milk supply.

The mothers appeared to be normally healthy but in some cases developed diarrhoea to a slighter or greater extent and the faeces were light in colour.

This question of the relationship between protein metabolism and milk secretion arose purely incidentally in the experiments. Its importance was at once recognised and therefore experiments to elucidate the problem were begun and are still in progress.

The mothers gained noticeably in weight and remained apparently in good health even when the young all died. Whether an excess of protein in the diet has any relation to the development of fits in suckling children or even

to the abnormal excitement and restlessness that is often seen in such children, remains to be examined by further investigation.

(*a*) *Effect of adding excess caseinogen to basal diet.* Water to drink (Fig. 1).

The proportions used were 15·0 g. bread and 5·0 g. caseinogen. The bread was well moistened and thoroughly mixed with the caseinogen.

The litter *P* did not develop the symptoms early, possibly because their mother ate less caseinogen than *N* or *T*. However three died; probably the others survived because they ate for themselves.

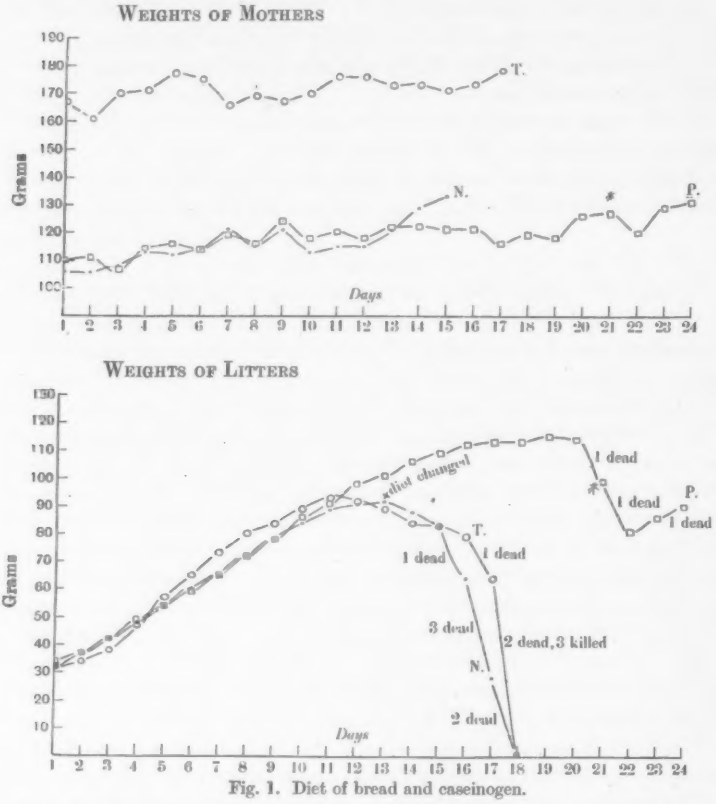


Fig. 1. Diet of bread and caseinogen.

Litter *N* all died, in spite of the fact that the mother's diet was changed to bread and milk on the 13th day when the symptoms became evident. It may be that the milk supply started again as found in experiment O (p. 154), but the babies were too weak to suck.

Litter *T* showed symptoms similar to those of litter *N*, one was found dead on the 17th day, two more died that day and the remainder were killed

together with their mother. The babies' stomachs were empty and their intestines almost so. The mother appeared normal, except that on cutting the mammary glands, no milk oozed out, but only a thin lymph-like secretion.

Result. Addition of excess caseinogen to the basal diet causes the mother to put on weight, but the milk supply is affected in such a way that the litters do well up to the 10th day, after which they cease to gain weight and finally die. It appears that the quality of the mother's milk, after being very good, is detrimentally altered before being completely suppressed.

(*b*) *Effect of adding caseinogen to other diets.* Water to drink (Fig. 2).

The diets given were:

1. Bread 15·0 g., caseinogen 5·0 g., meat extract 2·0 g. ... ... Exp. O
2. " " " " " " " butter 2·0 g. Exp. K
3. " " " " Yeast " " " " Exp. M

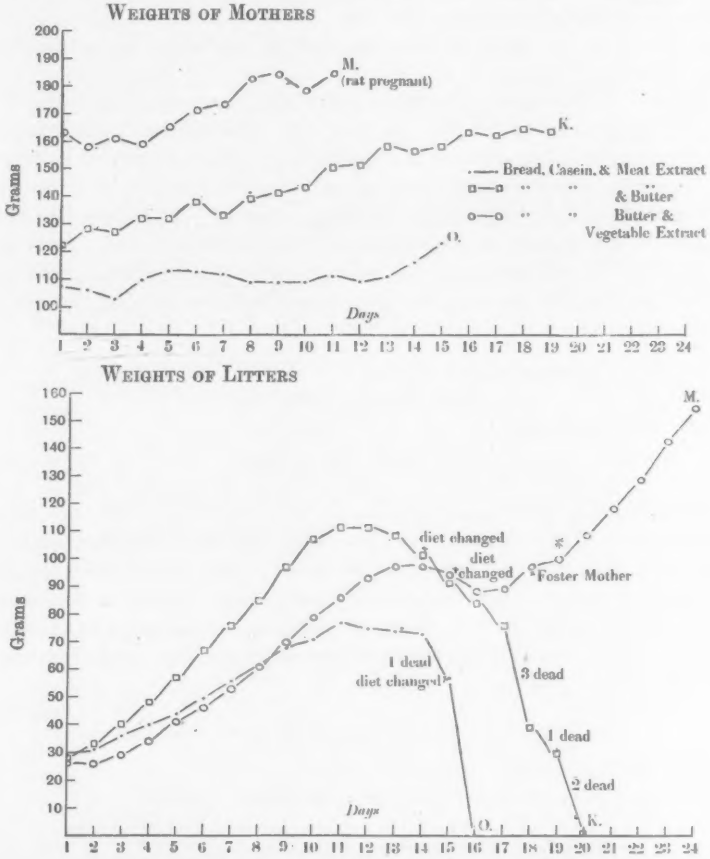


Fig. 2. Diet of excess caseinogen in different combinations.

The yeast extract used was "Marmite" and the foods were mixed as before described.

The mothers' weights again show a decided increase. The weighings of *M* were discontinued because she was found to be pregnant.

With regard to the litters, all the *K* babies died, in spite of the fact that the mother's diet was changed. It seems probable, as suggested previously, that the milk supply was not re-established until the babies were too weak to suck.

In the case of *M* the diet was changed to bread and milk before the symptoms became very severe, and as shown by the curve, the babies recovered. Later they were given to a foster mother as their own mother was pregnant and there was the possibility of the birth of another litter. (This second litter proved to be quite normal in every respect.) It may be that the rat's becoming pregnant again, while no others did so, is a factor which must be taken into account, but the work is insufficient to warrant any suggestions upon this point.

Litter *O* all developed the typical symptoms; one was dead on the 15th day and the remaining five died the next day. The mother *O* was killed on the 16th day (24 hours after the diet had been changed to bread and milk) and her mammary glands contained a small quantity of milk which led to the conclusion that the milk supply was just being restarted. The babies, like those of litter *N*, had nothing in their stomachs and very little in their intestines.

Result. The milk supply of the mother is stopped by excess caseinogen in any diet, but the secretion can be re-established by changing the diet to bread and milk.

(*c*) *Comparison of bread and milk diet with one containing excess caseinogen* (Fig. 3).

The diets used were:

1. 100 cc. milk, bread 15·0 g. (increased when necessary) ... Exp. D
2. Bread 15·0 g., caseinogen 5·0 g., meat extract 2·0 g., butter 2·0 g. ... Exp. K

An interesting point is brought out by this experiment. The two curves run parallel until the 10th day. This is a curious fact for it is to be remembered that bread and milk diet gives the best growth curve. After the 10th day, the litter *K* ceased to gain weight and finally died. Possibly a similar diet to that used in experiment K, *i.e.* bread, caseinogen, meat extract and butter, would give a maximum growth curve, if less caseinogen were used. It is hoped to work out this point later.

9. (*a*) *Comparison of animal and yeast extractives* (Figs. 4, 5).

The diets used were:

A. 1. Meat extract, bread, caseinogen, butter Exp. K } Fig. 4.
2. Yeast „ „ „ „ Exp. M }

B. 1. Meat extract, bread and butter ... Exp. I } Fig. 5.
2. Yeast „ „ „ ... Exp. A }

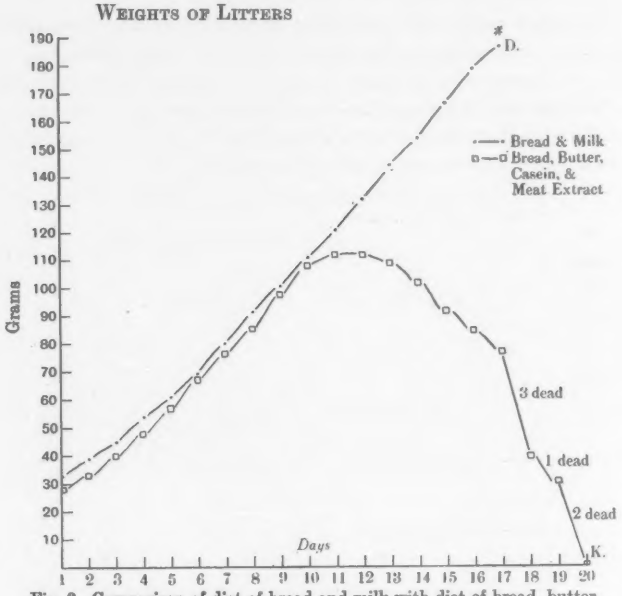


Fig. 3. Comparison of diet of bread and milk with diet of bread, butter, meat extract and caseinogen.

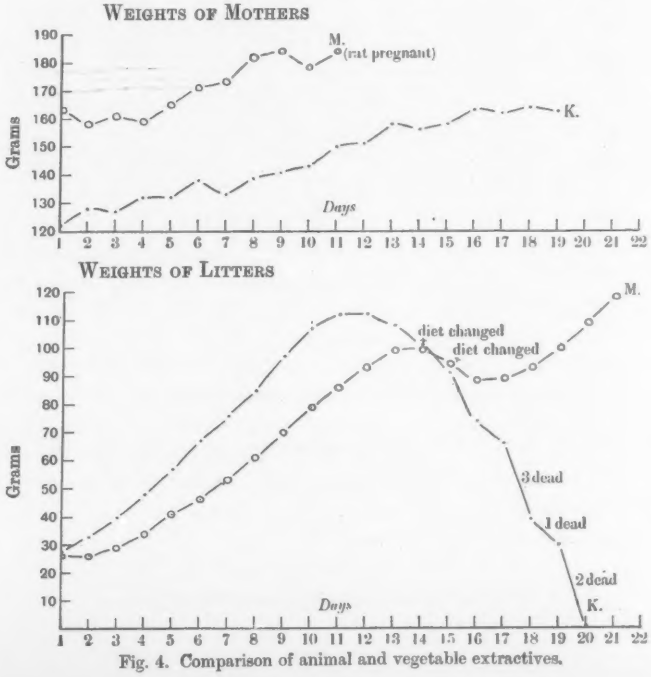


Fig. 4. Comparison of animal and vegetable extractives.

The diets were mixed as before described. The meat and yeast extracts used were the commercial preparations "Jardox" and "Marmite" respectively.

The work on this question is scanty, but tends to show that the growth curves of the young are better when the mother receives animal extractives, than when she is given yeast extractives in her diet.

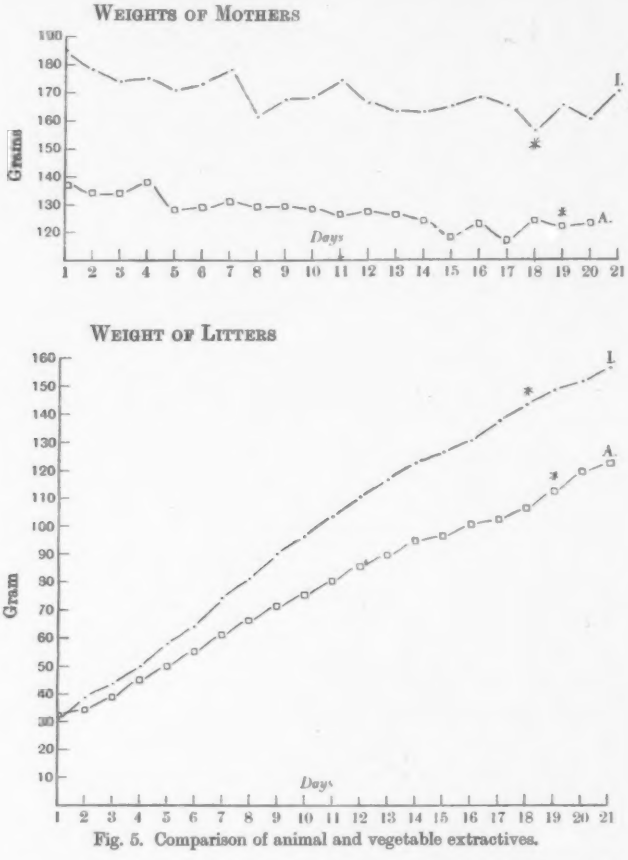


Fig. 5. Comparison of animal and vegetable extractives.

(*b*) *Comparison of various diets in relation to growth of litters* (Fig. 6).

In the following comparisons typical curves taken from the previously described experiments are used (see p. 158).

1. Comparison of (*a*) bread (basal diet),
   (*b*) bread and fat,
   (*c*) bread and additional carbohydrate.

The growth curves obtained when the mothers were fed on the above diets were almost identical. Hence fat, fat-soluble *A* and extra carbohydrate added to a poor diet appear to have no effect on the mammary secretion. The mothers show a distinct loss in weight during the lactation period.

2. Comparison of (*a*) bread (basal diet),
   (*b*) bread and meat extract,
   (*c*) bread and meat extract and fat.

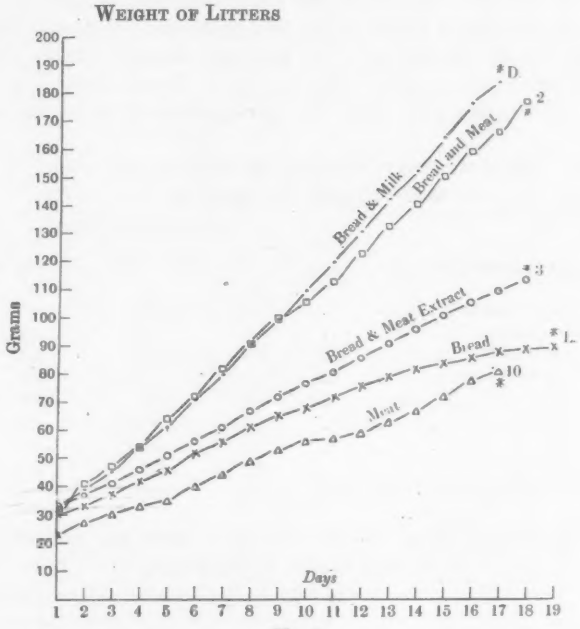


Fig. 6.

The typical "bread curve" is compared with curves obtained by feeding the mothers on bread and meat extract, and bread, meat extract, and butter respectively. This shows that better results are obtained with the improvement in diet and also that (as pointed out previously) butter has not very much effect. The difference in growth is so slight that it could even be obtained using the same diet, and it may be concluded that a diet of bread and meat extract is practically equivalent to a similar one with butter added so far as the secretion of milk is concerned.

3. Comparison of (*a*) bread (basal diet),
   (*b*) bread and meat,
   (*c*) bread and meat extract,
   (*d*) bread and milk,
   (*e*) meat only.

Fig. 6 represents a comparison of the typical growth curves obtained with widely different diets. To avoid complication in the figure, bread is taken as representing the curves of bread, bread and fat, and bread and additional carbohydrate, previously described as almost identical. Similarly bread, meat extract and fat is omitted owing to its resemblance to bread and meat extract with regard to effect on mammary secretion.

Comparing the diets in this way accentuates the fact that differences in the curves are greater towards the end of the lactation period, than at the beginning, *e.g.* the curves of (*D*) (bread and milk) and (2) (bread and meat) run together till the 9th day and then gradually diverge. Again the curve of *L* (bread only) runs parallel with that of (3) (bread and meat extract) until about the 7th day after which the extractives produce a better effect.

Table showing relative gain in weight of litter when mothers receive different diets.

Mother's diet	Average gain of litters		
	Days 1–7	Days 7–14	Days 14—eating
	g.	g.	g.
Bread only (basal diet)	4·5	3·6	2·2
Bread and butter	4·3	3·06	2·5
Bread and dextrin	4·5	3·25	2·0
Bread and meat extract...	5·1	5·25	5·0
Bread, meat extract and butter ...	5·3	6·1	4·7
Bread and meat	8·3	8·66	9·0
Meat only	3·5	3·2	4·5
Bread and milk	9·0	10·5	11·0
Bread, caseinogen, butter, meat extract	8·0	5·3	−8·0

Table showing initial and final weights of litters and mothers, when latter received different diets.

Mother's diet	Weights of litters		Weights of mothers	
	At birth	When eating for themselves	After birth of litter	When litter eating
	g.	g.	g.	g.
Bread only (basal diet) ...	30	90	120	105
Bread and butter	30	85	148	125
Bread and dextrin	30	88	173	144
Bread and meat extract ...	33	114	132	132
Bread, meat extract and butter	28	122	176	168
Bread and meat	26	143	140	140
Meat only	24	81	168	148
Bread and milk	35	185	163	161

(*c*) *The effect of change of diet during the lactation period* (Fig. 7).

The effect of changing the diet during the lactation period is most striking. It is obtained quickly, the difference in daily gain being noticeable within 24 hours after changing the food. The curve *G* represents the typical curve of a bread and milk diet and *L* that of a bread diet.

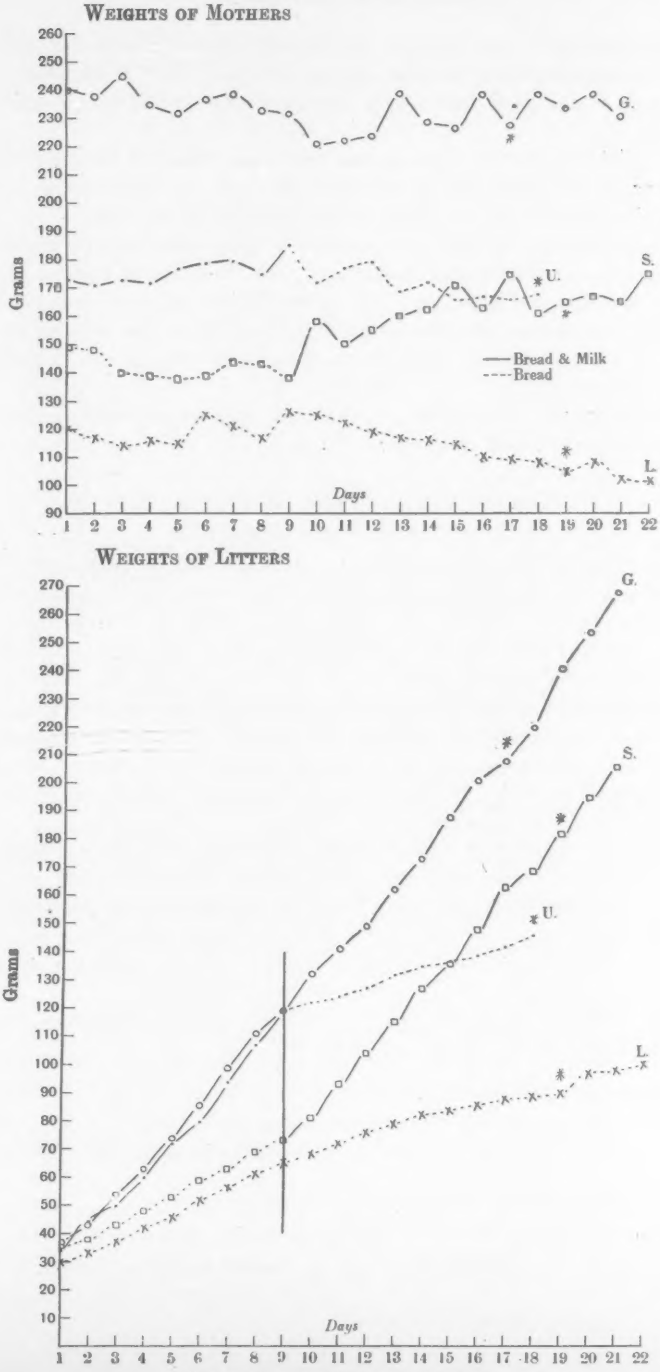


Fig. 7. Change of diet during lactation.

In experiment U the mother was fed on bread and milk for the first eight days and then the diet was changed to bread only. A decrease in the daily gain of the litter was noticed 24 hours after the change of diet (see Fig. 7).

In experiment S the converse was tried, *i.e.* bread for the first period, changing to bread and milk later and in this case the improvement in diet produced a corresponding increase in the daily gain of the litter.

It is interesting to note how the parts of the curves due to a bread diet run parallel with the typical bread curve, and those due to bread and milk diet with the typical bread and milk curve. This experiment indicates that it makes no difference whether the good diet be given at the beginning or the end of the lactation, since the milk supply is largely dependent on the daily intake of food.

This point was also noticed on changing from a diet composed entirely of meat to one of bread and meat.

IV. The supply of essential substances from the mother's tissues (Fig. 8).

Since the mothers receiving such inadequate diets as bread only, bread and dextrin etc., were able to bring up healthy litters, normal in all respects except that they were smaller than those of litters whose mothers received good diets, it seems evident that the mothers can supply various constituents for their milk from their own tissues.

The adequacy of the different diets was tested by keeping the young (after removal of the mother) on the same diets used during the lactation period. The results of these experiments are shown in Fig. 8. The day on which the babies began to eat for themselves is indicated by * and the removal of the mother is shown by an arrow.

From this diagram it is quite evident that with a full diet, *e.g.* bread and milk, bread and meat, the young will continue to grow at a fair rate after their mother has been taken away. On the other hand, with poor diets, the young rats gained weight very slowly after their mother had been removed.

From these "poor diet" results, it appears probable that the mother is able to supply certain food constituents absent from the diet.

The supply is cut short when the mother is removed and the young rats at once show a decline in the rate of growth.

Bread diet. Experiment L:

Baby rats gained 60 g. in 18 days when fed by mother.
" " 24 g. " 11 " " eating bread.

Bread and fat diet. Experiment Q:

Baby rats gained 68 g. in 17 days when fed by mother.
" " 18 g. " 14 " " eating bread and butter.

These experiments indicate that the mother can furnish from her own tissues, fat, fat-soluble *A*, water-soluble *B*, and the anti-scorbutic factor.

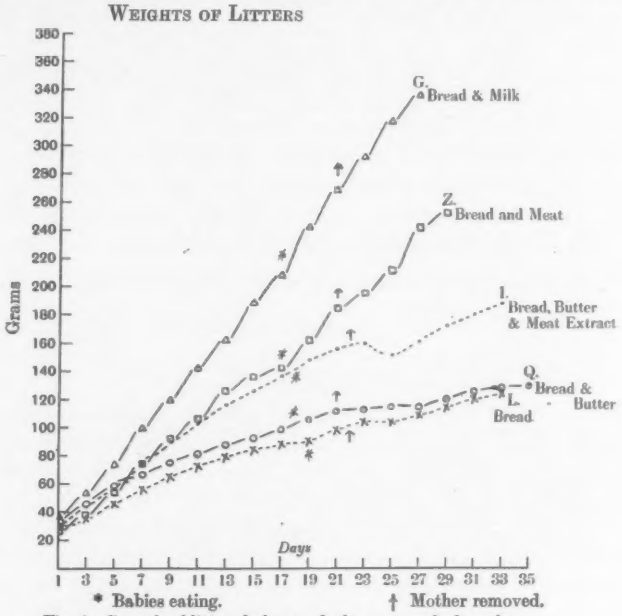


Fig. 8. Growth of litters before and after removal of mothers. Scale reduced ½ compared with other diagrams.

V. General Conclusions.

1. With certain typical diets, definite and different curves can be obtained for the rate of growth of the litters which obviously are an indication of differences either in quantity or quality or both of the mother's secretion of milk.

When the mother is given a good diet, *e.g.* bread and whole milk, the baby rats at the end of lactation weigh twice as much as when she receives a poor diet, *e.g.* bread only.

2. The loss or gain in the weight of the mother is not definite, but liable to great variation. Speaking generally the mother loses less weight as the diet contains more physiologically good elements. In the majority of cases the mother shows a typical initial loss of weight at the beginning of the lactation period.

3. Extractives tend to keep up the mother's weight. Protein has a similar effect.

4. Excess protein in the mother's diet probably alters the composition of the milk and after a time causes the milk supply to cease. On altering the diet to bread and milk, the secretion is again established.

5. Excess carbohydrate appears to have no effect at all.

6. Excess fat in the mother's diet has a slightly depressing effect on the growth of the litter, but the absence of fat seems to make practically no difference.

7. On any given diet, the litter belonging to a rat which eats a large amount, does better than one the mother of which eats a less amount.

8. The mother can supply certain essential substances from her own tissues, viz. fat, fat-soluble *A*, water-soluble *B* and the anti-scorbutic factor during the normal period of lactation.

9. The milk supply to a large extent is dependent on the food taken and can be increased or diminished within 24 hours by changing the diet.

The expenses of the research were partly defrayed by a grant from the Medical Research Committee.

REFERENCES.

Eckles (1911). *Dairy Cattle and Milk Production.*
Hart and Humphrey (1915). *J. Biol. Chem.* **21**, 239.
Hart, Nelson and Pitz (1918). *J. Biol. Chem.* **36**, 291.
Lusk (1917). *Science of Nutrition.*
McCollum, Simmonds and Pitz (1916). *J. Biol. Chem.* **27**, 33.

XVIII. INVESTIGATION OF THE ANTI-SCORBUTIC VALUE OF FULL CREAM SWEETENED CONDENSED MILK BY EXPERIMENTS WITH MONKEYS.

By ELEANOR MARGARET HUME.

From the Department of Experimental Pathology, Lister Institute.

(*Received January 5th, 1921.*)

Introductory.

Experiments have already been made [Barnes and Hume, 1919] on the anti-scorbutic value of raw, dried and heated cow's milk and for some of these experiments monkeys were used. Reference is there made to the literature of the subject, but as far as is known no experimental observations are extant on the anti-scorbutic value of sweetened condensed milk.

It is probable from what is already known of the anti-scorbutic value of milk heated to 100°, or above, that unsweetened condensed milk has lost all or almost all of its anti-scorbutic property, since it is heated at a temperature above 100° in order to sterilise it. With sweetened condensed milk however the high concentration of sugar is depended upon as the preservative after the preliminary pasteurisation of the milk. Concentration is carried out below 55° and is conducted *in vacuo*. It therefore seemed of interest to examine some well-known brand of condensed milk from this point of view and Nestlé's was chosen for the purpose.

In the work already referred to [Barnes and Hume, 1919], the minimum dose of raw milk daily needed to protect monkeys of 2–3 kilos. weight from scurvy was found to be 100–150 cc. daily. It was therefore only necessary to establish the minimum daily dose of condensed milk which would protect the same or similar animals from scurvy, in order to be able to calculate the loss, if any, suffered by the condensed milk.

Material used.

The material used consisted of two batches of milk, of which one batch was used in experiment 1 and the other in experiment 2.

By arrangement with the Nestlé Anglo-Swiss Condensed Milk Co., to whom the writer is indebted both for the gift of the material and for all details about it, the milk in each batch was absolutely uniform. Batch 1 was made

on June 7th, 1919, and the experiment was started in August, 1919; batch 2 was made on October 24th, 1919, and the experiment was started in February, 1920. In neither batch was any of the milk from stall fed cows.

The milk is condensed as follows:

Pooled full cream milk is first heated in a vessel with continuous inflow and outflow, the temperature of which is about 80°. It is computed that the milk remains at this temperature for about 3½ minutes. The sugar is next added; during this part of the process there is access of air. It then passes to a vacuum condenser where it boils at about 50° for about three hours and is drawn off when it passes a certain viscosity test. It is then cooled in bulk and during this part of the process there is again access of air.

Figures were provided by the Company giving the degree of condensation, which made it possible to calculate the amount of condensed milk equal to any given quantity of raw milk. These figures differ for every batch.

Technique of the Experiments.

The technique is fully set out in the paper already quoted [Barnes and Hume, 1919].

The basal diet consisted of boiled white rice and wheat germ and the ration of milk was relied on to provide sufficient vitamin *A*.

Both monkeys were male sooty mangabeys (*Cercocebus fuliginosus*) the anti-scorbutic needs of which had been previously ascertained. The one "Rags" had remained in health on a ration of 200 cc. of raw milk daily for 225 days, while the other "Toby" had developed scurvy after 77 days on a ration of 200 cc. dried milk; he was then cured on 200 cc. of raw milk, just scalded, daily. A ration of 200 cc. raw milk daily was therefore more than sufficient to provide anti-scorbutic for either of these monkeys, at a time when they weighed 2–3 kilos. In the course of the present experiments they grew to be monkeys weighing 5 and 4·5 kilos. respectively and were still growing; their anti-scorbutic needs might therefore be supposed to be greater and not less than in the preceding experiment.

Experimental Results.

Experiment 1. This experiment was carried out with the June batch of milk. Both monkeys received the equivalent of 250 cc. of fresh milk daily, Rags for 188 days and Toby for 199 days.

In this period Rags grew from 3370 g. to 4190 g., an increment of 820 g., while Toby grew from 3560 g. to 4320 g., an increase of 760 g. Both remained in perfect health the whole time.

Experiment 2. This experiment was conducted with the October batch of milk. Toby received the equivalent of 200 cc. of fresh milk for 260 days and increased from 4320 to 5020 g., an increase of 700 g. He was in perfect

health at the close of the experiment but the rate of growth was slower than in the previous experiment.

Rags received the equivalent of 150 cc. of fresh milk together with 50 cc. of the same milk, autoclaved at 120° for one hour; this destroyed the anti-scorbutic in the added 50 cc. but made the vitamin *A* value of the ration the same as in the case of Toby. The animal increased in 246 days from 4190 to 4570 g. an increase of only 380 g., less than half the growth in the former shorter period, and not much more than half the growth shown by Toby on the same vitamin *A* ration. The two animals' rates of growth in the first experiment on identical rations were almost exactly the same: the animal however appeared to be in perfect health at the close of the experiment.

The lag in Rags' growth on the 150 cc. ration might be due to a slight anti-scorbutic insufficiency, but since in previous experiments [Barnes and Hume 1919], the minimum requirement for monkeys of 2 to 3 kilos. was found to be 100 to 150 cc. of raw winter milk a day, it cannot be definitely stated that any loss of anti-scorbutic potency is demonstrated in the condensed milk.

The loss, if any, is small and the same is also true with regard to vitamin *A*. The present experiments were not made with a view to testing this property, but save for the wheat germ, the milk provided the sole source of vitamin *A* in the diet. Previous experience [Barnes and Hume, 1919] has shown that about 200 cc. of autoclaved milk daily provide a supply of vitamin *A* which enables good growth in monkeys of 2–3 kilos. to take place.

A recent experiment of Hopkins [1920] has shown that the vitamin *A* value of butter is only destroyed on heating at 120° for four hours, when access of air is permitted. Recent experiments by Delf [1920, p. 227] suggest that the anti-scorbutic vitamin is also much more susceptible to heat when free access of air is permitted. Unpublished experiments by Zilva show that there is loss of anti-scorbutic potency in substances exposed to the action of ozone. Doubtless these observations give the explanation of the non-destruction of vitamins in the condensation of Nestlé's sweetened condensed milk, for though the milk is heated for a very considerable time, though at a low temperature, it is heated *in vacuo*.

One important point should however be made with reference to the use of sweetened condensed milks in infant feeding. The above experiments show that there is little if any vitamin destruction in such milk but it must be borne in mind that in the experiments the milk was reconstituted to have as nearly as possible the same fat and vitamin (assuming no destruction) content as raw milk. That is to say that it was diluted at most four times (one vol. condensed milk to about three vols. water) whereas instructions for the use of the milk for infants suggest a dilution of at the lowest eight times and at the highest very much more. So large an addition of water produces a dilution of the fat and vitamins to an extent likely to be dangerous, although the high

percentage of sugar gives an adequate calorie supply, a danger to which attention was drawn by Coutts [1911].

Thanks are due to Dr C. J. Martin and to Dr H. Chick for help in directing the experiment and to Miss H. Henderson Smith for help in caring for the monkeys.

REFERENCES.

Barnes and Hume (1919). *Biochem. J.* **13**, 306.
Coutts, F. J. H. (1911). *Local Govt. Board Report.* Food Reports No. 15.
Delf (1920). *Biochem. J.* **14**, 211.
Hopkins (1920). *Brit. Med. J.* ii, 147.

XIX. NOTE ON THE PERMEABILITY OF THE RED CORPUSCLES FOR AMINO-ACIDS.

By SHUZO KOZAWA and NOBU MIYAMOTO.

From the 2nd medical clinic, Medical College, Osaka.

(*Received January 13th, 1921.*)

In previous publications, one of the authors, S. Kozawa [1914, 1919], has shown that the red corpuscles of man and monkey can take up all the hexoses and pentoses, as well as glucosamine. This was proved by chemical analysis and by means of the hematocrit. In connection with this experiment, the permeability of red corpuscles was also tested for amino-acids by means of the hematocrit, and the following results were obtained on the blood of different animals.

Table I.

Time	Alanine	Glycocoll
I. *Red corpuscles of man.*		
20 minutes	27·2	27·2
6 hours	28·5	27·5
II. *Red corpuscles of dog.*		
15 minutes	29·0	—
5 hours	27·5	—
III. *Red corpuscles of horse.*		
10 minutes	28·2	—
2 hours	29·0	—
IV. *Red corpuscles of ox.*		
10 minutes	25·5	24·0
5 hours	24·0	23·0

These results seemed to show that the red corpuscles were impermeable for amino-acids. The hematocrit test is, however, not so exact as chemical methods. It has been stated by Constantino [1913], who employed the formol method of estimation, that glycocoll, asparagine and the amino-acids obtained by the hydrolysis of caseinogen penetrated in small amounts into the red corpuscles of ox blood. We, therefore, thought it worth while to reinvestigate the permeability of the corpuscles in man, ox, and goat for amino-acids, employing the method of van Slyke [see Plimmer, 1915] for our estimation. For the removal of blood protein, we employed the method of Rosenberg [1914], using a 2 % solution of mercuric chloride, containing 0·8 % HCl.

Experimental.

The amino-acids used in these experiments were isotonic solutions of glycocoll (2·2 %), and histidine (4·7 %). We first determined the quantities of the amino-acids present in normal defibrinated blood and its constituents, namely, the red corpuscles and serum. We then added 0·2 g. of each amino-acid (2·2 % glycocoll solution, 9·0 cc.; 4·7 % histidine solution, 4·25 cc.) to each 30 cc. of defibrinated blood. The mixture was then placed for one hour in an incubator at 38°. The red corpuscles and serum were then separated by means of a powerful centrifuge. The red corpuscles separated in this way still contain a small quantity of serum, which is estimated by means of the hematocrit, and allowed for in the formol estimation. After one hour in the incubator, 5 to 10 cc. of the mixture are taken up with a pipette, the proteins are removed, as already indicated, and the nitrogen contents estimated by the van Slyke method. By this method, glycocoll would give rather larger figures than are actually obtained, but this has been allowed for by reducing the results in the ratio of 105 to 100.

Table II.

I. *Experiments on ox blood.*

A. Normal defibrinated blood.

	Kind of blood	Volume of amino-nitrogen (corrected) cc.	Amino-nitrogen in 10 cc. mg.
I.	Defibrinated blood 20 cc.	2·40	0·6936
	Red corpuscles 20 cc. ...	2·15	0·6256
	Serum 20 cc.	2·80	0·7989
II.	Defibrinated blood 20 cc	3·23	0·9173
	Red corpuscles 20 cc. ...	2·80	0·7952
	Serum 20 cc.	3·18	0·8991
III.	Defibrinated blood 20 cc.	3·80	1·1105
	Red corpuscles 20 cc. ...	3·50	1·0228
	Serum 20 cc.	3·20	0·9884

B. Defibrinated blood 30 cc. with 0·2 g. glycocoll or histidine.

	Kind of blood	Volume of amino-nitrogen (corrected) cc.	Further corrected with hematocrit volume cc.	Amino-nitrogen in 10 cc. mg.
I.	Red corpuscles with glycocoll 10 cc.	6·93	3·135	1·8386
	Serum with glycocoll 10 cc. ...	22·10	—	12·9285
II.	Red corpuscles with histidine 10 cc.	5·04	3·50	1·9687
	Serum with histidine 10 cc. ...	11·20	—	6·3000
III.	Red corpuscles with histidine 10 cc.	5·17	3·88	2·2756
	Serum with histidine 10 cc. ...	10·34	—	6·0644

II. *Experiments on goat's blood.*

A. Normal defibrinated blood.

Kind of blood	Volume of amino-nitrogen (corrected) cc.	Amino-nitrogen in 10 cc. mg.
Red corpuscles 10 cc. ...	2·66	1·5324
Serum 10 cc.	1·98	1·1414

B. Defibrinated blood with amino-acid.

	Kind of blood	Volume of amino-nitrogen (corrected) cc.	Further corrected with hematocrit volume cc.	Amino-nitrogen in 10 cc. mg
I.	Red corpuscles with glycocoll 10 cc.	8·84	5·13	2·9420
	Serum with glycocoll 10 cc. ...	23·66	—	13·5350
II.	Red corpuscles with histidine 10 cc.	6·60	5·60	3·2284
	Serum with histidine 10 cc. ...	10·64	—	6·1339

III. *Experiments on human blood.*

A. Normal defibrinated blood.

Kind of blood	Volume of amino-nitrogen (corrected) cc.	Amino-nitrogen in 10 cc. mg.
Blood 5 cc. ...	1·16	1·36996
Red corpuscles 5 cc. ...	1·17	1·39814
Serum 10 cc.	2·20	1·31450

B. Defibrinated blood with amino-acid.

	Kind of blood	Volume of amino-nitrogen (corrected) cc.	Further corrected with hematocrit volume cc.	Amino-nitrogen in 10 cc. mg.
I.	Red corpuscles with glycocoll 10 cc.	10·08	7·10	4·1748
	Serum with glycocoll 10 cc. ...	21·84	—	12·8184
II.	Red corpuscles with histidine 10 cc.	6·88	5·02	2·9317
	Serum with histidine 10 cc. ...	14·30	—	8·3512

It is evident from the results of these tables, that the amino-acid content of the red corpuscles undergoes a considerable increase on the addition of amino-acids to the whole blood. In the experiments given, the corresponding content of amino-acids was raised to two or three times the normal amount, and was below the content of the serum. We are thus, by the application of van Slyke's method, able to confirm, on ox, man and goat, the results obtained by Constantino in ox blood by the formol method.

Conclusion.

We may conclude that the red corpuscles of ox, man and goat are, to a certain extent, permeable for amino-acids, such as glycocoll and histidine.

REFERENCES.

Constantino (1913). *Biochem. Zeitsch.* **55**, 411.
Kozawa (1914). *Biochem. Zeitsch.* **60**, 231.
—— (1919). *J. Physiol.* **53**, 264.
Plimmer (1915). *Practical Organic and Biochemistry.*
Rosenberg (1914). *Biochem. Zeitsch.* **62**, 157.

XX. THE ESTIMATION OF BLOOD SUGAR.

By ERIC PONDER and LAURENCE HOWIE.

From the Department of Physiology, University of Edinburgh.

(*Received January 13th, 1921.*)

The method devised by Folin and Wu in 1918 and revised by them in 1920 [1918, 1920] has afforded a means of estimating blood sugar with a high degree of consistency and accuracy. To obviate the one objection to this method—namely, the necessity of using a larger quantity of blood than is readily obtained from a finger prick—Mackenzie Wallis and Gallagher [1920] have devised a micro-method, based partly on the work of I. Bang [1914], but chiefly on the method of Folin and Wu. That their method is convenient in the respect that small quantities of blood are required, is beyond doubt: their technique, however, introduces several very undesirable features, besides being somewhat lengthy. In their method the small quantity of blood required is weighed on absorbent paper on a torsion balance: the glucose is then extracted from the paper by distilled water, the proteins precipitated by tungstic acid, and the sugar in the filtrate estimated by the method of Folin and Wu.

The use of the torsion balance is an unfortunate feature of this method, for it almost necessarily introduces an initial error of considerable magnitude, owing to the evaporation from the absorbent paper. We have performed a series of weighings to demonstrate the extent of this error as follows: the subject was bled directly on to a piece of absorbent paper of known weight, hanging on the torsion balance, and the weight of the blood obtained by rapid weighing, the weighing being completed within ten seconds after the withdrawal of the blood from the finger. The piece of paper with the absorbed blood was then left hanging on the balance, and repeated weighings made at the end of one, two, three, and four minutes. The progressive loss of weight, which was greatest during the first minute, was found to be fairly constant for all samples of blood. That this loss by evaporation should occur is unavoidable, and only to be expected: when it occurs to the extent of several milligrams in a sample of only about 150 mg. weight, the percentage error is too great to be disregarded.

We give a table of the results obtained with five weighings—these five being representative of a large number made (the balance used was an instrument by Hartmann and Braun).

Weight of blood	Loss % occurring in			
	1 min.	2 mins.	3 mins.	4 mins.
163	1·2	1·8	3·0	3·0
163	1·8	2·4	2·4	3·7
165	1·8	3·0	3·6	3·6
165	1·8	2·4	3·0	3·6
160	1·9	2·5	3·1	3·7
Mean	1·7	2·4	3·0	3·5

It will be seen that if the interval between the withdrawal of the blood from the finger of a patient and the completion be as long as one minute an error of nearly 2 % is introduced, whereas if the interval be longer, a greater error will be present. This is unsatisfactory. Mackenzie Wallis and Gallagher prefer this method to the use of small sized pipettes of doubtful accuracy; since, however, it is an easy matter to calibrate a pipette to deliver 0·2 cc. with an error of as little as 0·5 %, we have preferred the use of such to the use of the torsion balance. The use of pipettes further greatly simplifies the technique, and at the same time avoids an obvious fallacy in the micro-method of these authors, the assumption that all the glucose in the blood is extracted from the absorbent paper by distilled water; it also avoids the inconvenient lengthening of the time required for the estimation, which this extraction entails.

It may be pointed out that the error introduced by the use of the torsion balance (tending to give too high a percentage), may be compensated by an incomplete extraction of the glucose from the absorbent paper, the results finally obtained perhaps agreeing with results given by the method of Folin and Wu. To rely on this occurring is, however, most undesirable, for this compensation may not regularly occur.

We suggest the use of the following simple micro-method, which is essentially the method of Folin and Wu applied to small quantities.

Special apparatus, etc. required:

1. The solutions required for the precipitation of proteins and the estimation of sugar in blood by the method of Folin and Wu.

2. Small boiling tubes, about 7 × 70 mm. The necks of these tubes are drawn out so as to form a tube of about 10 mm. long and 3 mm. in diameter. The tubes must be able to contain 1·2 cc., but not more than 1·5 cc.

3. A micro-filtration apparatus. A small funnel about 1 inch in diameter, is passed through a rubber stopper in a wide, flat-bottomed tube, carrying a side tube which may be attached to a filter pump. The funnel delivers the filtrate into a small tube, about 10 × 30 mm. which is contained inside the tube above described. Over the filter is a cover, to protect the blood from evaporation during filtration. Slight suction only is used, applied not constantly, but intermittently, and only occasionally; the papers used with the filter are "extra-hardened." This filter will produce 0·6 cc. of filtrate from the amount of blood used.

4. Pipettes, accurately calibrated by weight, to deliver 0·4 cc. and 0·2 cc. These may be operated by teats.

The estimation is done as follows:

1. Blood is drawn from the finger into a 0·2 cc. pipette. The contents of the pipette are added to 1 cc. of distilled water: the pipette is then twice filled with water, and these volumes, carrying with them the traces of blood from the walls of the pipette, are added to the tube containing the blood and distilled water. 0·2 cc. of sodium tungstate and 0·2 cc. of 2/3 *N* sulphuric acid are then added: the tube is allowed to stand for five minutes at least, its contents being occasionally shaken gently.

2. The contents of the tube are placed in the filter; after about two minutes' filtration through hardened paper under slight pressure, sufficient filtrate will be obtained. It is important to keep the filter covered during filtration.

3. To a small boiling tube, as above described, is added 0·4 cc. of a 0·01 % glucose solution; while to a similar tube is added 0·4 cc. of the filtrate. To each is added 0·4 cc. of the alkaline copper tartrate solution. The contents are mixed by stirring. The narrow tube-like necks of the boiling tubes are sealed rapidly in a blow pipe flame, care being taken not to heat the contents of the tube. The air space left will be small if the tubes are of the proper size. The tubes are immersed in a boiling water bath for six minutes.

4. At the end of this time, the temperature of the bath is lowered to about 80°. The necks of the tubes are cut off, and 0·4 cc. of the phenol reagent are added to each tube; this requires care lest the contents of the tubes be expelled. The tubes are allowed to cool. Each is emptied into a tube containing 3·8 cc. of water, thus making the volume of the contents of this tube up to 5 cc.

5. The contents of these tubes are matched in the colorimeter in the usual way; and the percentage of glucose present in the blood calculated.

This method has in our hands given results closely comparable with the results given by the method of Folin and Wu. The following table shows the results obtained in ten specimens of blood examined (*a*) by the method of Folin and Wu, and (*b*) by the method above described.

	% of sugar	
Specimen	Folin and Wu	Micro-method
1	0·121	0·123
2	0·103	0·102
3	0·093	0·094
4	0·055	0·054
5	0·148	0·148
6	0·125	0·122
7	0·150	0·151
8	0·145	0·142
9	0·127	0·127
10	0·138	0·139

It should be noted that these are not all samples of normal blood under the usual conditions, since certain of them were taken after prolonged fasting, while others were taken after excessive carbohydrate intake.

It is claimed for this modification that (1) the amount of blood required may readily be obtained from the finger, (2) that this amount of blood is accurately known, and that none of it is lost by manipulation, (3) that re-oxidation during boiling is reduced to a minimum, (4) that the colour finally obtained in the "blood" tube is very similar to that in the "standard," and (5) that the results obtained are practically identical with those given by the method of Folin and Wu.

We have to thank Professor Sir Edward Sharpey Schafer for giving facilities for the carrying out of this research; our thanks are also especially due to Dr W. W. Taylor, for the advice which he has so readily given.

REFERENCES.

Bang (1913). *Biochem. Zeitsch.* **49**, 1.
—— (1914). *Biochem. Zeitsch.* **57**, 300.
Folin and Wu (1918). *J. Biol. Chem.* **38**, 106.
—— (1920). *J. Biol. Chem.* **41**, 367.
Mackenzie Wallis and Gallagher (1920). *Lancet*, Oct. 16.

XXI. THE EFFECT OF ACETALDEHYDE AND METHYLENE BLUE ON THE FERMENTATION OF GLUCOSE AND FRUCTOSE BY YEAST-JUICE AND ZYMIN IN PRESENCE OF PHOSPHATE AND ARSENATE.

By ARTHUR HARDEN and FRANCIS ROBERT HENLEY.

From the Biochemical Department, Lister Institute.

(*Received January 18th, 1921.*)

It was previously shown by the authors [Harden and Henley, 1920, where the literature is quoted] that, in the fermentation of glucose in presence of phosphate by zymin or yeast-juice, acetaldehyde or methylene blue produced a remarkable acceleration in the early stages of the reaction and it was suggested that the function of these substances was to act as hydrogen acceptors and thus enable the reaction at once to attain a high rate. The experiments have now been extended to fructose, with the objects of ascertaining the maximum rates attainable with or without added acceptors and of comparing these with the corresponding rates yielded by glucose.

I. Comparison of the maximum rates and the course of the reaction observed in the presence of phosphate with glucose and fructose respectively in the presence and absence of acetaldehyde.

A. *Zymin.* To 2 g. of zymin were added 10 cc. of a solution containing 1 g. of the sugar and, when acetaldehyde was used, 3 cc. of 1 % acetaldehyde; 0·2 cc. toluene was added in each case. This mixture was incubated at 25° until a constant rate of fermentation had been attained and varying volumes of 0·6 *M* K_2HPO_4 (previously saturated with CO_2) added. Readings were in many cases made every two minutes but the rates have usually been calculated for five minutes. With the smaller quantities of phosphate repeated additions were made when the rate began to slacken. This procedure was adopted because, especially in the case of glucose, the rate was depressed even by a small excess of phosphate. The final volumes therefore occasionally differ slightly, but this has very little effect on the maximum rate obtainable.

In Table I are given the maximum rates obtained with and without acetaldehyde, and the volumes of phosphate solution added.

Table I.

		With 3 cc. 1 % acetaldehyde		Without acetaldehyde	
No.	Sugar	Max. rate cc. per 5 min.	Additions	Max. rate cc. per 5 min.	Additions
1	Fructose	15	1 of 5 cc.	7·25	1 of 5 cc.
2	„	14	2 of 2 „	9	2 of 2 „
3	„	14	3 of 1 „	9·75	3 of 1 „
4	Glucose	9	2 of 2 „	7	1 of 2 „
5	„	7·5	2 of 1 „	5·5	1 of 1 „
6	„	9	1 of 2 „	—	—
7	„	8·25	1 of 2 „ and 1 of 1 „	—	—
8	Cane-sugar	8·75	1 of 2 „	7·5	2 of 2 „

B. *Yeast-juice.* The experiments with yeast-juice were carried out in a similar manner to the above, 20 cc. of yeast-juice and 4 cc. of 1 % acetaldehyde being used, the total volume being 24·6 cc.; 0·2 cc. of toluene was added in each case. The results are embodied in Tables II and III, each of which refers to a different sample of yeast-juice.

Table II.

		With 4 cc. 1 % acetaldehyde		Without acetaldehyde	
No.	Sugar	Max. rate cc. per 5 min.	Additions	Max. rate cc. per 5 min.	Additions
9	Glucose	16	1 of 1 cc.	10·75	3 of 1 cc.
10	„	18·5	1 of 1 „ and 1 of 2 „	11·75	2 of 2 „
11	„	21	1 of 5 „	—	—
12	Fructose	29·6	1 of 5 „	24·5	1 of 5 „
13	„	36	1 of 10 „	33	1 of 10 „

Table III.

14	Fructose	17·25	1 of 5 cc.	—	—
15	„	18·25	4 of 2 „	—	—
16	Cane-sugar	8·25	1 of 5 „	5·5	1 of 5 „
17	„	11·25	2 of 2 „	10	1 of 2 „

Owing to the fact that repeated additions of phosphate were made in most of these cases, the course of the reaction was irregular. Fig. 1 however shows the characteristic effect exerted by acetaldehyde on the fermentation of fructose in presence of phosphate (experiment B 13 above). In this case the rates in cc. per 2 min. are plotted against the time, and it is seen that in the presence of aldehyde the rate rises much more rapidly, but only to a slightly higher maximum.

It will be seen from the above that the maximum rate obtainable from glucose under the optimum conditions of phosphate and aldehyde falls far short of that observed with fructose. For the sample of zymin employed for experiments A 1–8 the maximum rates in presence of aldehyde were 15

for fructose and 9 for glucose, a ratio $F/G = 15/9 = 1{\cdot}67$. In the case of the yeast-juice used in experiments B 9–13 the ratio $F/G = 36/21 = 1{\cdot}7$, almost identical with that for zymin.

It appears to follow from this, since all these rates were determined in presence of an excess of acceptor, that the difference in the behaviour of fructose and glucose although partly attributable to a difference in the rate of production or of reduction of acceptor, cannot be wholly accounted for in this way, but must extend to some other stage in the reaction.

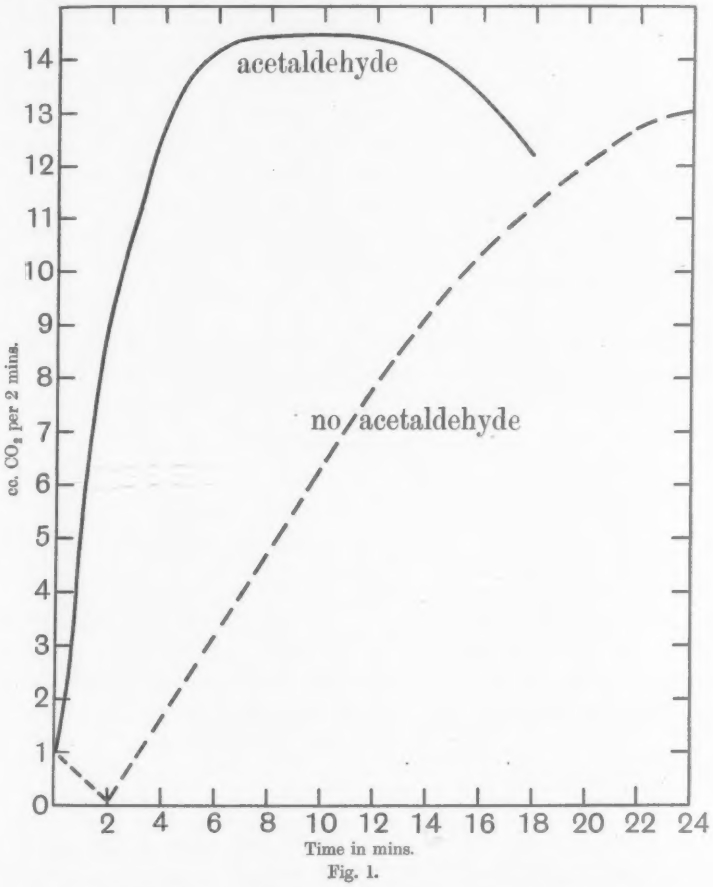


Fig. 1.

II. Relative efficiency of acetaldehyde and fructose as agents of "induction."

It was suggested in the previous paper [Harden and Henley, 1920] that the inductive effect of fructose [Harden and Young, 1909] on a mixture of

glucose and phosphate was due to the more ready production of acceptor from fructose than from glucose. This might occur in either or both of two ways: firstly, by the more rapid production from fructose of a substance capable of acting as an acceptor in the preliminary stage of the reaction (possibly identical with the precursor of glycerol and possibly a different substance from that produced from glucose) or by the more rapid reduction of such a preliminary acceptor under the prevailing conditions; secondly by the more rapid fermentation of the fructose, yielding as intermediate product acetaldehyde (which may be termed the normal acceptor) which would then be available to complete the cycle of fermentation of further molecules either of fructose or of glucose, even in the presence of a considerable excess of phosphate. Under these circumstances it became of interest to ascertain the relative efficiencies of fructose and acetaldehyde in this respect. Experiments were therefore made with yeast-juice and glucose in presence of excess of phosphate by adding varying amounts of acetaldehyde and fructose and comparing their effects, with the result that a concentration of 1/42,200 of acetaldehyde gave almost exactly the same reaction curve as 1/211 of fructose (see Fig. 2), this latter being equivalent to 10 % of the glucose present. In other words acetaldehyde is 50 times as effective as fructose, when molecular quantities are compared.

Experiment 18. Six quantities of 25 cc. yeast-juice + 2 g. glucose + 10 cc. 0·6 *M* K_2HPO_4 + 0·2 cc. toluene were incubated for ten minutes at 25° and then were added:

No.	10 % glucose cc.	10 % fructose cc.	1 % acetaldehyde cc.	Water cc.
1	2	0	0	4
2	2	0	0·1	3·9
3	2	0	0·5	3·5
4	2	0	1·0	3
5	2	0	1·5	2·5
6	0	2	0	4

The course of the fermentations in the various flasks is shown by the curves of Fig. 2, rates in cc. per five minutes being plotted against time. The curve for (4) was almost coincident with that for (5), whilst that for (3) was intermediate between those for (4) and (2). It will be seen that the addition of 0·1 cc. of acetaldehyde solution (2) produced almost exactly the same effect as that of 2 cc. of the fructose solution (6).

III. Effect of acetaldehyde on the fermentation of glucose and fructose by yeast-juice and zymin in presence of arsenate and phosphate.

It has previously been shown [Harden and Young, 1911] that arsenate greatly accelerates the action of the hexosephosphatase of yeast-juice and zymin so that the fermentation of glucose and fructose proceeds much more rapidly in the presence of a suitable concentration of arsenate than in its

absence. Excess of arsenate, however, produces inhibition. It therefore became of interest to ascertain whether the arsenate, in addition to its effect on the hexosephosphatase, exerted any modifying influence on any other stage of the fermentation and in particular whether it in any way affected the accelerating action of acetaldehyde, a possibility which has not previously been considered.

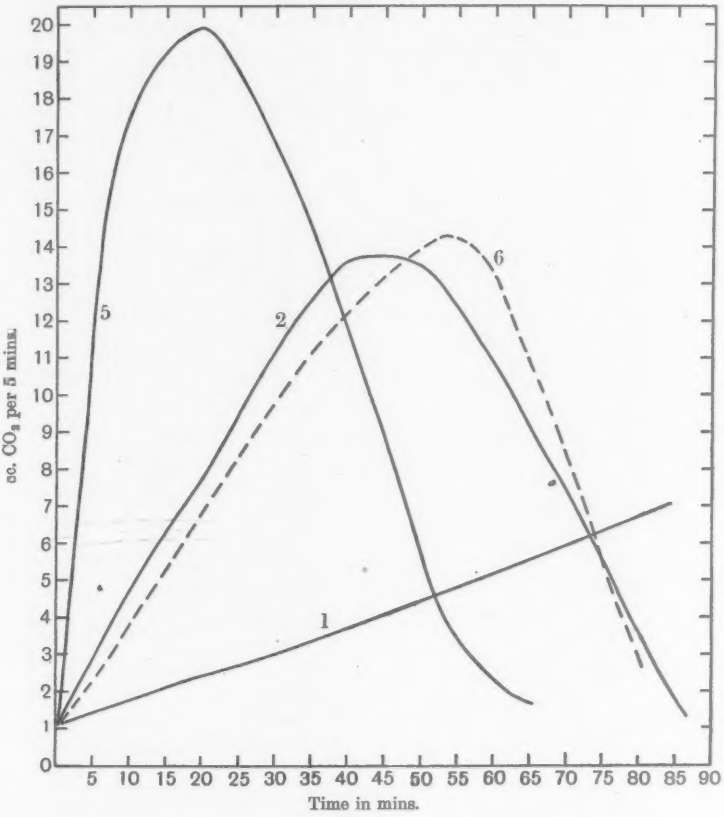


Fig. 2.

C. *Yeast-juice.*

Experiment 19. Six flasks were made up as follows:

No.	Yeast-juice cc.	Sugar	Toluene cc.	Water cc.	1% acetaldehyde cc.	0·3 *M* Na_2HAsO_4 cc.	0·6 *M* K_2HPO_4 cc.
1	20	2 g. fructose	0·2	15	0	0	5
2	20	,,	0·2	10	5	0	5
3	20	,,	0·2	14	0	1	5
4	20	,,	0·2	9	5	1	5
5	20	,,	0·2	10	0	5	5
6	20	,,	0·2	5	5	5	5

The sodium arsenate and potassium phosphate were added to the mixture after it had been incubated for 30 mins. at 25°. The course of the fermentation in the different flasks is shown by the curves of Fig. 3.

A similar experiment was carried out with glucose, a different sample of yeast-juice being employed, which was capable of producing fermentation in presence of an exceptionally high concentration of phosphate.

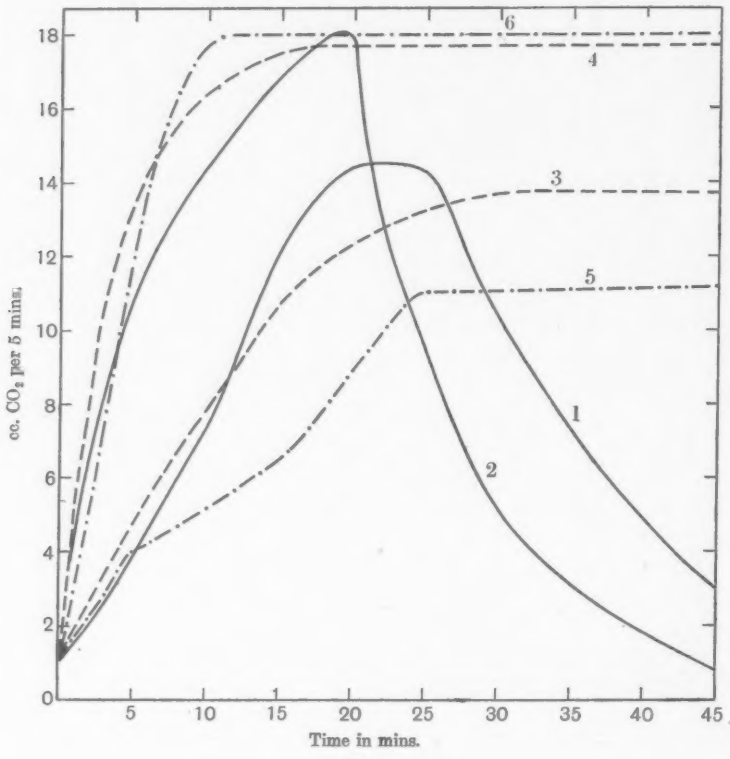


Fig. 3.

Experiment 20. Six flasks were made up, each containing 20 cc. yeast-juice + 2 g. glucose + 0·2 cc. toluene together with:

No.	Water cc.	1 % acetaldehyde cc.	0·3 *M* Na_2HAsO_4 cc.	0·6 *M* K_2HPO_4 cc.
7	12·5	0	0	7·5
8	7·5	5	0	7·5
9	11·5	0	1	7·5
10	6·5	5	1	7·5
11	7·5	0	5	7·5
12	2·5	5	5	7·5

The sodium arsenate and potassium phosphate were added to the mixture after it had been incubated for 30 mins. at 25°.

The course of the fermentation was very similar to that observed in experiment 19 with fructose, as is indicated by the following observations:

No.	Arsenate cc.	Acetaldehyde cc.	Maximum rate cc. per 5 min.	Time (min.) required to attain max.
7	0	0	18	60
8	0	5	33·6	10
9	1	0	16·8	85
10	1	5	35·9	10
11	5	0	(7·1)	90
12	5	5	22·7	10

The small increase of rate in 10 as compared with 8 is probably due to the fact that in 8 the optimum concentration of phosphate was not present, whereas in 10, owing to the action of the arsenate, optimum conditions were maintained.

Two interesting points emerge from these experiments:

1. In presence of excess of phosphate, arsenate does not remove the inhibition, but somewhat increases it (Curves 1, 3, 5, Fig. 3 and experiment 20, Nos. 7, 9, 11).

2. The presence of arsenate does not affect the accelerating action of acetaldehyde in presence of phosphate (Curves 3, 4, 5 and 6, Fig. 3 and experiment 20, Nos. 8, 10).

D. *Zymin.*

Experiment 21. Twelve flasks were made up each containing 2 g. zymin with the following additions; 0·2 cc. of toluene was added in each case:

				Added subsequently		
No.	Sugar	Water cc.	1 % acetaldehyde cc.	0·6 *M* K_2HPO_4 cc.	0·3 *M* Na_2HAsO_4 cc.	Water cc.
1	1 g. glucose	9·4	0	2	0	1·2
2	„	6·4	3	2	0	1·2
3	„	9·4	0	2	0·1	1·1
4	„	6·4	3	2	0·1	1·1
5	„	9·4	0	2	0·2	1
6	„	6·4	3	2	0·2	1
7	1 g. fructose	9·4	0	3	0	0·2
8	„	6·4	3	3	0	0·2
9	„	9·4	0	3	0·1	0·1
10	„	6·4	3	3	0·1	0·1
11	„	9·4	0	3	0·2	0·2
12	„	6·4	3	3	0·2	0·2

The course of the reaction is indicated by the following observations:

No.	Sugar	Arsenate cc.	Acetaldehyde cc.	Maximum rate cc. per 5 min.	Time (min.) required to attain maximum
1	Glucose	0	0	5·9	40
2	,,	0	3	6·2	10
3	,,	0·1	0	6·8 (average)	45
4	,,	0·1	3	9·6 ,,	10
5	,,	0·2	0	6·9 ,,	45
6	,,	0·2	3	9·9 ,,	10
7	Fructose	0	0	9·8	35
8	,,	0	3	12·1	10
9	,,	0·1	0	10·1 (average)	35
10	,,	0·1	3	14·1 ,,	10
11	,,	0·2	0	9·9 ,,	40
12	,,	0·2	3	14·4 ,,	10

In its main features the zymin behaves in a similar manner to yeast-juice. Owing to the small concentration of arsenate employed, the inhibiting effect of the arsenate, which was marked in the case of yeast-juice, is here barely perceptible.

Another point of difference is that the maximum rate, especially with glucose, in presence of arsenate is considerably greater than in its absence, the two rates in question being 6·2 and 9·9 or a ratio of 1·6, whereas with yeast-juice and glucose the ratio is 33·6/35·9 = 1·07. The difference is much less with fructose, the corresponding ratios being 1·2 for zymin and 1·0 for yeast-juice. In order to ascertain whether a real difference of this kind exists between yeast-juice and zymin, experiments were made in which the maximum rate in presence of the optimum concentration of phosphate in presence of acetaldehyde was determined, and arsenate was then added to the same mixture. With glucose this produced, as in the previous experiment, a marked rise of rate, whereas with fructose only a small and insignificant rise was observed. This shows that the arsenate exerts a definite accelerating effect on the fermentation of glucose by zymin apart from its action on the hexose-phosphatase. The exact significance of the phenomenon has not yet been ascertained.

Experiment 22. 2 g. zymin + 1 g. of the sugar (glucose or fructose) + 3 cc. 1 % acetaldehyde + 6·44 water + 0·2 cc. toluene were incubated at 25° until the rate was constant. Successive small additions of phosphate were made, as in experiments 1–8, until the maximum rate obtainable had been observed. 0·2 cc. of 0·3 *M* Na_2HAsO_4 was then added and the average rate attained was noted.

	Maximum (cc. per 5 min.)	
Sugar	In absence of arsenate	In presence of arsenate
Glucose	6·0	8·5
Fructose	13·0	13·5

IV. Effect of methylene blue.

It was shown in the previous communication [Harden and Henley, 1920] that methylene blue behaved in a similar manner to acetaldehyde with respect to the fermentation of glucose by zymin in presence of phosphate. These experiments have now been extended to the fermentation of both glucose and fructose in the presence of phosphate, with the result that the methylene blue has been found to behave in a similar manner in all these cases.

The experiments were carried out exactly as in the case of acetaldehyde, but the methylene blue was added in the solid form. No attempt was made to ascertain the optimum conditions, so that the rates observed are not necessarily the maximum obtainable.

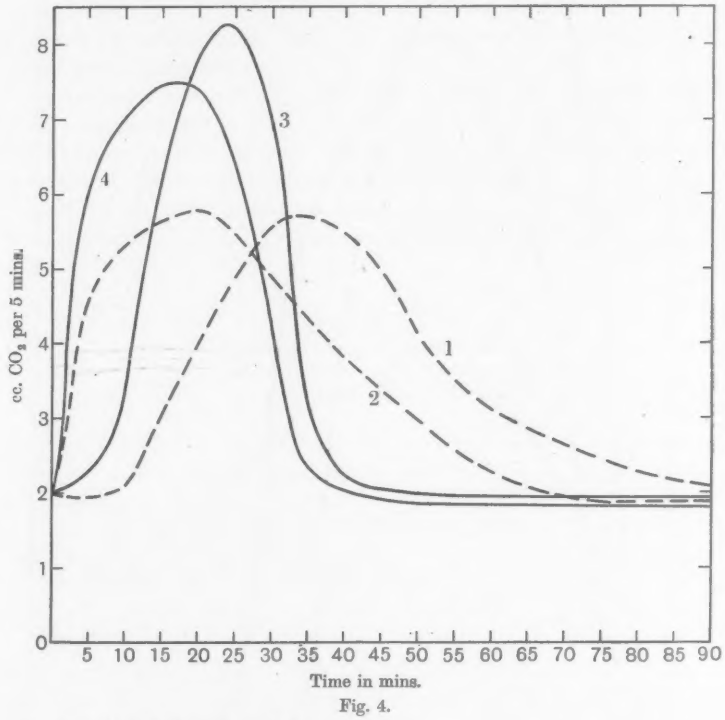


Fig. 4.

E. *Zymin and glucose and fructose.*

Experiment 23. Four flasks were made up containing each 2 g. zymin + 1 g. of sugar + 9·4 cc. water + 0·2 cc. toluene. Nos. 1 and 2 contained glucose, Nos. 3 and 4 fructose. 0·1 g. methylene blue was added to Nos. 2 and 4. These were incubated for 35 mins. at 25° and 2 cc. 0·6 *M* K_2HPO_4 were then added to each. The course of the reaction is seen from the curves (Fig. 4).

F. *Yeast-juice and glucose and fructose.*

Experiment 24. Four flasks were made up containing

1. 25 cc. yeast-juice + 2 g. glucose + 0·2 cc. toluene
2. „ „ + „ + „ + 0·2 g. methylene blue
3. „ „ + 2 g. fructose + „
4. „ „ + „ + „ + „ „

These were incubated for 15 mins. at 25° and 15 cc. 0·6 *M* K_2HPO_4 were then added. The course of the reaction is shown in the accompanying curves (Fig. 5).

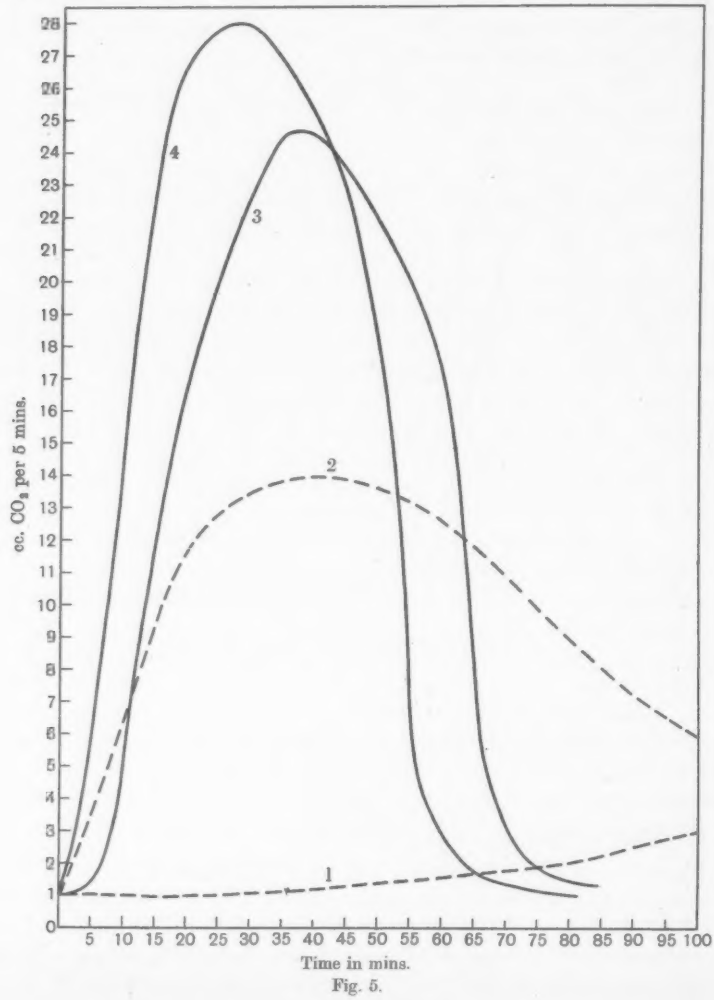


Fig. 5.

In every case the addition of the methylene blue caused a marked acceleration in the rate of attainment of the maximum. The concentration of the phosphate was so high as greatly to inhibit the fermentation of the glucose in absence of methylene blue (curve 1).

Summary.

1. Acetaldehyde diminishes the time required by a mixture of fructose and phosphate to attain its maximum rate of fermentation in presence of yeast-juice or zymin, but does not substantially increase the maximum rate obtainable.

2. In presence of acetaldehyde fructose is more rapidly fermented than glucose in presence of phosphate both by yeast-juice and zymin.

3. Acetaldehyde is about 50 times as effective as fructose (when molecular quantities are compared) in "inducing" fermentation in a mixture of glucose with excess of phosphate.

4. Arsenate does not affect the accelerating action of acetaldehyde in presence of phosphate in the fermentation of glucose and fructose by yeast-juice but considerably increases the rate attained with glucose, and to a less degree that attained with fructose, when zymin is used.

5. Methylene blue produces an effect similar to that of acetaldehyde in the fermentation of both fructose and glucose by zymin and yeast-juice in presence of phosphate.

REFERENCES.

Harden and Henley (1920). *Biochem. J.* **14**, 642
Harden and Young (1909). *Proc. Roy. Soc.* B. **81**, 336.
—— —— (1911). *Proc. Roy. Soc.* B. **83**, 451.